

ENZYME REACTORS IN ANALYTICAL DETECTION SYSTEMS THEORY AND APPLICATIONS

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THEORY AND APPLICATIONS

by

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Enzyme Reactors in Analytical Detection Systems. Theory and Applications.

Abstract

Methods for the analysis of samples of clinical interest for urea, L-aminoacids, glucose, cholesterol or oxidized cholesterol analogues, were developed. Environmental samples can be analyzed for inorganic- and organic-mercury, by high specific enzymatic reactions. Activators like zinc can also be determined. Reactors, filled with enzymes immobilized on porous glass are utilized in the detection systems. The theory for the design of enzyme reactors for different methods are discussed. Separation of the enzyme and the detector in the flow system offers the possibility to optimize the conditions for both. Flow-techniques as plug-flow, flow-injection analysis (FIA) and post-column reactions have been used. Adaption to clinical samples have been made by using on-line dialysis. Complete conversion within the reactor makes the method insensitive to moderate variations in physical parameters or enzyme activity. The selectivity of the detectors used, ammonia-gas-membrane electrode and spectrophotometric detection combined with the selectivity inherent in the enzymatic reaction gives a very high overall system selectivity.

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This thesis is a summary of the following papers which will be referred to in the discussion by roman numerals I - VII.

- I An Enzyme Reactor Electrode for Urea Determinations
Gillis Johansson and Lars Ögren,
Anal. Chim. Acta, 84(1976)23-29
- II An Enzyme Reactor Electrode for Determination of Amino Acids
Gillis Johansson, Kerstin Edström and Lars Ögren,
Anal. Chim. Acta, 85(1976)55-60
- III Application of Ion-Selective Electrodes to Enzymatic Analysis
Gillis Johansson and Lars Ögren,
in Ion-Selective Electrodes, Second Symposium held at Mátra-
füred, Hungary, 18-21 October, 1976, ed. by E. Pungor,
Akadémiai Kiadó, Budapest, 1977, p. 93-100
- IV Flow Injection Analysis of Glucose and Urea Using Enzyme
Reactors and On-Line Dialysis
Lo Gorton and Lars Ögren,
Submitted to Anal. Chim. Acta
- V A Post-Column Enzyme Reactor for Detection of Oxidized
Cholesterols in HPLC Separations
Lars Ögren, Istvan Csiky, Lars Risinger, Lars-Göran Nilsson
and Gillis Johansson,
Anal. Chim. Acta, 117(1980)71-79

VI Determination of Traces of Mercury(II) by Inhibition of
an Enzyme Reactor Electrode Loaded with Immobilized Urease

Lars Ögren and Gillis Johansson,

Anal. Chim. Acta, 96(1978)1-11

VII Determination and On-Site Sampling of Inorganic and Organic
Mercury in Aqueous Samples with Enzyme Reactors

Lars Ögren,

Anal. Chim. Acta, 125(1981)45-53



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1. INTRODUCTION

Since the first isolation of an enzyme in pure form, urease, by Sumner¹ in 1926, the progress has been tremendous. More than 2000 enzymes are known today². A few hundred of these are commercially available. Enzymes are classified according to the type of reaction they catalyze. The nomenclature² distinguish six classes.

1. Oxidoreductases
2. Transferases
3. Hydrolysases
4. Lyases
5. Isomerases
6. Ligases (Synthetases)

Analytical uses has been developed as the functions and properties of the enzymes have been more thoroughly understood. A great number of methods are now available for measuring enzyme activities³ or for the determination of substrates⁴. Many of the methods are of great importance for clinical diagnostics and for medical research³.

The technique for enzyme immobilization has been developed in the last decade and is now well established⁵⁻⁷. A recommended nomenclature for immobilized enzymes has been given by Sundaram et al⁸.

- | | |
|--------------|----------------------|
| 1. Entrapped | a) Matrix-entrapped |
| | b) Microencapsulated |
| 2. Bound | a) Adsorbed |
| | b) Covalently bound |

The insolubility obtained by the immobilization gives advantages in many analytical applications and can be further developed in

new analytical systems. A number of different approaches have been described in the literature and some of them are already in use in commercial instruments.

An enzyme electrode is a sensor obtained by covering an electrode with a layer of enzyme constrained by a dialysis membrane or a membrane consisting of cross-linked enzyme. The enzyme electrode brings together both the separation function, namely the membrane, the enzyme and the detector in a simple compact unit.

With the concept of enzyme reactors or tubes the site of the reaction is normally physically separated from the detection system. The reactor consists of a tube filled with enzyme immobilized on a support, preferably non-compressible. In the tube the enzyme has been immobilized on the walls. Enzyme reactors and their advantages will be discussed in detail later. A number of other approaches have also been developed e.g. the enzyme stirrer⁹, the enzyme thermistor probe¹⁰, the enzyme chemiluminescent probe¹¹, solid-surface fluorometry¹² and stirred solutions with immobilized enzyme. Analytical applications, mostly of clinical interest, have been summarized^{4, 7, 13-16} and reviewed¹⁷⁻¹⁹.

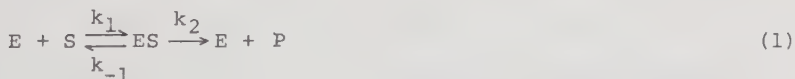
2. THEORY OF ENZYME REACTOR DESIGN

The theoretical behaviour of enzyme electrodes in steady-state²⁰ and during transient conditions²¹ have been studied. Their design, however, makes them sensitive to physical variations and changes in enzyme activity¹³. At a high level of enzyme activity or with a thick enzyme layer the sensitivity is dependent only on the mass-transfer and on the diffusion coefficient of the substrate and the product. If the substrate concentration is too high ($[S] \gg K_m$),

the response becomes independent of the bulk concentration of the substrate^{13,15}. Unlike enzyme electrodes, an enzyme reactor should be designed to give a complete conversion of substrate over a large concentration range. To measure inhibitors or activators with enzyme reactors other constructions are utilized.

2.1 Enzyme reactor for substrate conversion

The kinetics of enzyme reactors has been studied by several authors²²⁻²⁴. Usually the treatment presupposes a Michaelis and Menten kinetics²⁵. Even if such a mechanism applies only to some enzyme systems the equations below can be used as an approximation of the performance of the reactor for many other enzyme systems. The reaction mechanism used to describe the enzymatic reaction is



in which the substrate, S, forms a complex, ES, with the enzyme, E. This complex is in turn splitted into a product, P and the free enzyme.

Integration of the kinetic equation, leads to a relation between the length, l , of the reactor and the conversion efficiency, η , (paper III).

$$l = \frac{q}{V_{\max} \cdot A} [S_0 \cdot \eta - K_m \cdot \ln(1-\eta)] \quad (2)$$

where A is the reactor area and q the volumetric flow-rate. The concentration of substrate entering the reactor, S_0 , is related to the concentration of the product, P, through $\eta = P/S_0$.

$V_{\max}$ (the optimal reaction rate for the immobilized enzyme) and K_m (the Michaelis-Menten constant for the enzyme reactor), are obtained as described in paper III ($K_m = K_s$). They are dependent on the flow-rate²² and other physical parameters²² as well as on the enzyme loading (paper VII). They should be of the same order of magnitude as $V_{\max}$ and K_m for the soluble enzymes. In designing enzyme reactors three alternatives are possible:

I. $S_0 \gg K_m$. In this case, eqn (2) reduces to $l = \text{const} \cdot S_0 \cdot \eta$ and there is a linear correlation between the reactor length and the conversion efficiency. For a 100% conversion, i.e. $\eta = 1$, the equation gives the maximum substrate concentration which still gives full conversion.

II. $S_0 \ll K_m$. Eqn (2) then becomes $l = \text{const} \cdot [-K_m \cdot \ln(1-\eta)]$.

The conversion is independent of the substrate concentration and the relation between conversion and length is:

Conversion/%:	50	90	95	99	99.9	99.99
Length(relative):	0.7	2.3	3.0	4.6	6.9	9.2

III. $S_0 \approx K_m$. In this case, there is a mixed contribution from substrate concentration, conversion factor and K_m . Eqn (2) is then used with all its terms. For a reactor with limited conversion, deviation from linearity is observed for higher substrate concentrations.

In an enzyme reactor it is advantageous to achieve a 100% conversion efficiency and it is even desirable to operate with a certain over-capacity to make up for subsequent losses in enzymatic activity.

For a group-specific enzyme the relative enzymatic activity varies for different substrates. Theoretically the conversion in an enzyme reactor is found to be more favourable than expected from the relative activity in solution. This has been shown theoretically for an enzyme reactor, in a report²⁶ (Table 1).

Table 1. A comparison between the relative activity in solution and the conversion within the enzyme reactors for different analogue activities, $S_0 = K_M/20$.

Relative analogue activity in solution/%	Analogue activity in reactor/%	
	for $\eta = 0.99$	for $\eta = 0.90$
100	99	90
50	90	68
30	74	49
10	36	20
5	20	11

2.2 Measurement of inhibitors

In enzyme reactors for determination of an inhibitor a completely different approach has been used (paper VI). Under the same assumptions as in 2.1, but introducing the effect of inhibition in the eqn (2), the following equation is obtained (paper VI, eqn (6)).

$$e_i \cdot A \cdot \bar{L} = \frac{S_0 \cdot q}{k_2} [\eta_e - \eta_i + \frac{K_m}{S_0} \ln(\frac{1-\eta_i}{1-\eta_e})] \quad (3)$$

The left-hand side of eqn (3) is proportional to the amount of inhibitor. η_e and η_i correspond to the conversion efficiency for a fully active, respectively, partly inhibited reactor. k_2 is the rate constant in the eqn (1).

Computer plots (paper VI, Fig. 1) show that the highest sensitivity is obtained for a high substrate concentration at a given K_m . For high values of η_e there is an increased curvature in the response curves, when $\frac{K_m}{S_0}$ increases. A low enzyme loading will of course also increase the sensitivity.

2.3 Measurement of activators

A theoretical evaluation of the parameters affecting an enzyme reactor for measuring activators (for instance essential metals) has been described in a report²⁷. The following equation applies,

$$e_o \cdot A \cdot \bar{L} \cdot \frac{k_2}{q} = P - K_m \ln(1-\eta) \quad (4)$$

where $e_o \cdot A \cdot \bar{L}$ correspond to the amount of active enzyme in the reactor. The enzyme activity is zero in the absence of metal. When a small amount of a metal, a , is added the reactor will be partly reactivated and the quantity of active enzyme is $e_a \cdot A \cdot \bar{L}$. The amount of added metal is related to the amount of active enzyme by

$$a \cdot k = e_a \cdot A \cdot \bar{L} \quad (5)$$

where the constant, k , is dependent on the number of metal atoms per enzyme molecule. A rearrangement and combination of eqns(4) and (5) gives

$$a/S_0 = k' \cdot \left(\eta_a - \frac{K_m}{S_0} \ln(1 - \eta_a) \right) \quad (6)$$

where k' is a constant for the enzyme reactor used, and η_a is the conversion factor for the activated reactor. Data simulation using eqn (6) is shown in Fig. 1.

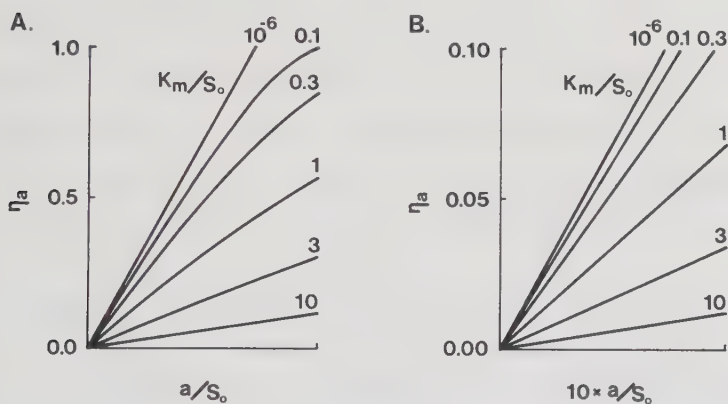


Fig. 1. Computer simulated plots of the activation of an enzyme reactor with immobilized apoenzymes. A) represents the complete activation of a reactor and B) a reactor activated up to at most 10%.

From this data it is inferred that the highest sensitivity is obtained for $K_m/S_0 < 0.1$. Curved calibration curves is another drawback although, as shown in Fig. 1 B, for the region limited by values of $\eta < 0.1$ straight lines are observed. The physical configuration of the reactor should be of no importance as long as it is sufficiently large to give time for the metal to react with the apoenzyme in the reactor. This step can, however, be kinetically limited.

3. PROPERTIES OF ENZYME REACTORS

3.1 Packing support

A good support for immobilization of enzymes for analytical applications should have a good flow performance, a high mechanical stability and bind a large amount of enzyme. Inorganic supports such as glass, silica and alumina have high stability and good flow through characteristics¹³. Porous supports increase the available surface. The surface and the binding capacity increase with decreasing pore diameter with an optimum of 300 Å for an enzyme with m.w. 48000 D²⁸. The studies in this thesis were carried out on porous glass, with pore diameter of normally 700 Å and a particle size of 120-200 mesh. Sieved glass with a narrower mesh-fraction was used to improve flow performance (paper VII).

The glass is normally very stable except at high pH, where hydrolysis of the silicate may cause a loss of enzyme. The stability can be increased by coating the porous glass with ZrO_2 ²⁸.

Alcohol dehydrogenases immobilized on ZrO_2 -stabilized glass were found to be twice as stable at pH 9.0 as on an untreated glass²⁷. High stability at neutral pH indicates that the rate of hydrolysis for the porous glass is low (papers I-III, V-VII).

3.2 Immobilization technique

The immobilization technique pioneered and described by Weetall²⁸ was employed throughout. It is one of the simplest and most general methods of coupling⁶. The carrier is silanized giving free amino groups. A difunctional reagent, glutaraldehyde couples to the carrier and the excess is washed away before the enzyme is added.

Lysine and terminal amine residues of the enzyme react with the aldehyde and the immobilization is thus accomplished. Other reactions are also possible, as described by Weetall²⁸.

3.3 Enzyme loading

A maximum loading gives the best performance for a reactor with substrate conversion. Using 100 mg of enzyme per gram of glass a loading of 13 mg (peroxidase) to 46 mg (glucose oxidase) is obtained (paper IV). For more exclusive (and expensive) enzymes lower amounts were used, however, as well as for methods utilizing the enzyme reactor for measuring inhibitors (paper VI and VII).

3.4 Stability

Besides the effect of hydrolysis of the carrier, enzyme denaturation also affects the reactor stability. This is a temperature dependent process, although operation at 37°C for prolonged time does not seriously alter the reactor performance (papers II and V).

Purified enzymes, show high stability (papers II and V) but crude preparations lost their activity within a few days. Urease, for the measurement of urea (paper I) was used during one month without any apparent stability change. Sudden activity losses were, however, detected (paper VII) as already observed by Lee et al²⁹.

3.5 Conversion efficiency

A high conversion efficiency ($\approx 100\%$) makes an enzyme reactor free from influence of moderate variation in physical parameters or enzyme activity³⁰. These requirements were fulfilled for urease (papers I and IV), L-aminoacid oxidase (paper II), glucose oxidase and peroxidase (paper IV).

Cholesterol and oxidized cholesterols were not completely converted (60-72%) due to the high alcoholic content of the solution (paper V). The calibration curve for cholesterol deviates slightly from linearity as expected from theory as the $K_{m(\text{app})}$ value (10^{-4}M) of the immobilized enzyme is larger than the substrate concentration. As shown theoretically (2.1) the conversion of a substrate-analogue, with relatively low activity in relation to the main substrate, is higher within the enzyme reactor. This was confirmed experimentally (Table 1, paper V) for sterols and oxidated sterols.

A high equilibrium constant of the reaction is required for a complete conversion. This was the case in the studies presented, except for L-alanine dehydrogenase-alcohol dehydrogenase (L-ADH, ADH) (paper III). Equilibrium was achieved for L-ADH but not for ADH. The detector response was dependent on the flow rate (Fig. 5, paper III). For inhibitor measurement a low conversion is preferred (papers VI and VII).

3.6 Cosubstrate requirement

To get an acceptable enzymatic activity and to approximate an enzymatic reaction with a Michaelis-Menten-reaction the supply of cosubstrate should not be a limiting factor. Oxidases demand an oxidating cosubstrate, usually oxygen. The concentration of oxygen in an air saturated solution ($3 \cdot 10^{-4}\text{M}$) is insufficient for high substrate concentrations. In the reaction catalyzed by L-aminoacid oxidase (paper II), catalase was also immobilized in the enzyme reactor to increase the oxygen concentration by conversion of hydrogen peroxide. To further expand the measuring range hydrogen peroxide was added to the buffer. The oxygen supply to

glucose oxidase (paper IV) and cholesterol oxidase (paper V) was maintained by saturating the solutions with oxygen.

One of the reasons of the difficulties noted with the multi-enzyme system (paper III) was probably caused by the low co-enzyme concentration. The enzymatic detection of H_2O_2 (paper IV) is also limited at high H_2O_2 concentrations by the reagent concentration.

3.7 Reactor material

In applications involving the measurement of enzyme activity it is of great importance that the reactor material does not affect the enzyme. Different plastic materials and glues affected urease but an all-teflon construction did not inhibit the enzyme. A change from silver frits to woven polypropylene filter (papers IV, V and VII) gave better flow performance to the reactors.

3.8 Effect of variation of physical and chemical parameters

The enzymatic reaction rate is affected by the following chemical and physical parameters.

- a) substrate concentration
- b) enzyme concentration
- c) activators
- d) inhibitors
- e) temperature
- f) pH
- g) ionic strength

As stated in 3.5 a reactor with a complete conversion (and some overcapacity) is unaffected by moderate variations in b)-g). It was shown experimentally that variations in the flow rate, enzyme concentration, temperature (paper I) or pH (pH = 7-8, paper II) did not affect the performance of high conversion reactors.

Low conversion systems, like cholesterol oxidase (paper V), showed dependence on flow-rate, temperature and alcoholic content, among others. These parameters had thus to be carefully controlled. To eliminate the sensitivity to temperature of the reactors for inhibition measurements (papers VI and VII), two different procedures for thermostating the reactors are described.

4. DETECTORS

The use of enzyme catalysed reactions for analytical purposes is conditioned by the availability of methods for measuring either the generated or the consumed compounds. The species most commonly measured in order to follow an enzyme reaction are listed in Table 2, and the measuring principles for the detection are given.

Other detection principles of more general nature are thermometry (thermistor), to follow reactions with high exothermic production, and methods to measure the production or consumption of H^+ .

Table 2. Analytical methods for measuring species most commonly utilized to follow enzyme reactions.

Measured species	Class of enzyme*	Detection principle
NAD(P)H	1	a) Spectrophotometer (340 nm) b) Fluorescence c) Amperometry d) Colorimetric detection with redox indicator
O ₂	1	Clark electrode (amperometrical)
H ₂ O ₂	1	a) Amperometry b) Chemical reaction giving species measured by I Colorimetry II Fluorometry III Amperometry IV Potentiometry V Chemiluminescence
NH ₃ , NH ₄ ⁺	1,3,4,6	a) Ion-selective electrode (potentiometry) b) Gas sensitive electrode c) Colorimetric detection with chemical reagents d) Coupling to a NADH-consuming reaction
ATP	a few of 2,6	Bioluminescence
CO ₂	4,6	Gas sensing electrode
PO ₄ ³⁻	6	Colorimetrically by chemical reagents

*The numbers refer to the classes of enzyme listed in Introduction

Enzyme electrodes are restricted to amperometric and potentiometric detection. This limits the method to a few classes of enzymes. Even if the detection can be made electrochemically, this method may be disadvantageous compared to the other detection methods. The reactor on the other hand gives much larger freedom in the selection of a detection system.

As a rule, detection systems based on chemical reactions (not enzyme catalyzed) increase the interferences from the matrix and are normally the time consuming step in the flow system. Below are described the ammonia-probe, one of the most specific detectors, and the spectrophotometer detection, the most general detector to the species listed above.

4.1 Gas-sensing membrane probe

When ammonium is the product of the enzymatic reaction, an ammonia-gas sensing membrane probe was used. It is a complete electrochemical cell consisting of a pH-glass electrode in an ammonium chloride solution and a reference electrode. The pH-sensitive tip of the electrode is pressed against a gas-permeable membrane separating the electrolyte from the sample solution, forming a thin film. The sample is made alkaline ($\text{pH} > 11.3$) and the ammonia in the sample diffuses through the membrane until the partial pressures in the film and in the sample are equal. The resulting pH-change in the film is measured. The response is logarithmic in the range $10^{-5}\text{M} - 10^{-1}\text{M}$ of ammonia.

The performance of this type of probes has been investigated by Bailey and Riley³¹⁻³³. Temperature variations and high ionic strength cause potential drift. Therefore the probe is thermostated and the ionic strength is kept below 0.2 M (paper I).

The probe is not affected by alkali metals in contrast to the ammonium selective glass electrode³⁴ or the ammonium selective nonactin electrode³⁵. The only species interfering are some volatile bases^{36,37}. As a result of its high selectivity, the combination of an enzyme reactor and a NH_3 -probe is very suitable for direct measurement in samples with complicated matrixes.

A relatively long response time makes the electrode to the rate limiting element for a high sample through-put system both in steady-state measurements (papers I-III, VI and VII) and in Flow Injection Analysis (FIA) (paper IV). Other principles for the detection of the pH-change in the thin film have been used³⁸. The employment of an air-gap electrode eliminates the clogging of the membrane when working with clinical samples³⁹.

4.2 Spectrophotometer

Spectrophotometric detection is a very commonly used method for many enzymatic reactions. The specificity of the method depends on the wavelength, on the molar absorptivity of the measured species and on any interfering species. Interferences can be reduced by splitting the flow before the reactor and pass one part through a reference cell and the other part through the sample cell after the enzymatic reaction. In steady state method the reactor can be

placed between a reference and a measuring flow-through cuvette, compensating for the absorbing species in the sample.

In an optimized FIA-system with a low dispersion or in a HPLC-system where the enzyme reactor is used as a post-column component (paper V) the resolution between the peaks has to be maintained and this is achieved by using a low dead volume HPLC-detector.

When oxidases are used, the solutions are first saturated with oxygen at elevated temperature, and then cooled. This prevents the formation of micro-bubbles which otherwise might interfere with the spectrophotometric detection (paper IV and V).

5. APPLICATIONS

5.1 Flow systems for the detection of substrates

5.1.1 Plug-flow

The performance of plug-flow enzyme reactors is illustrated for two enzyme systems, a simple one, urease, and a co-substrate demanding enzyme, L-aminoacid oxidase.

Urea

Analysis of urea is of considerable interest in clinical chemistry for diagnosis of kidney malfunction, in agricultural chemistry, where urea is used in fertilizers, in environmental pollution control and many other applications⁴. The enzyme immobilized was urease (EC 3.5.1.5) catalysing the reaction

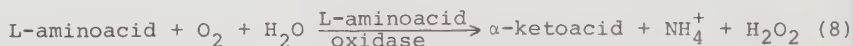


High specificity of the enzyme, selectivity of the ammonia-electrode and system optimization through complete conversion in the reactor and independent selection of pH in the reactor and the electrode (see Fig. 1, paper I), give an almost selective method for urea for a wide range of concentrations (Fig. 2, paper I). The response is not affected by moderate variations of a number of chemical or physical factors. Interference from ammonium in the samples is easily prevented by the insertion of an ion-exchange column in the sample line prior to the reactor. Direct measurements or standard additions procedures show excellent agreement when dilute urine samples are analyzed. The reproducibility was better than 2%.

A urea analyser based on these principles is now commercially available^{40,41}. Other detection systems like microcalorimetry⁴² and cation-sensitive electrodes⁴³ have also been used with urease reactors. Enzyme electrodes with ammonium selective electrodes^{34,35} are prone to interferences from alkali metals in the samples. Enzyme electrodes based on the ammonia gas electrode have also been used⁴⁴ in order to overcome ionic interferences. The selection of pH for the system must be a compromise between that required by the enzyme and that of the electrode. Enzyme electrodes based on a pH-electrode⁴⁵ also overcome ionic interferences and operate well within the pH-range suitable for the enzymes. The response will be affected by changes in the buffer capacity of the sample solutions.

L-aminoacid

Determination of L-aminoacids is of clinical importance⁴. In paper II a system for determination of L-aminoacids is described, using L-aminoacid oxidase (EC 1.4.3.2), a flavoprotein, as a catalyst for the reaction



The enzyme, which has a complex reaction pattern, (eqns (1)-(4), paper II) shows, in spite of this, good characteristics in the reactor. The range is limited by the supply of oxygen, but using immobilized catalase and H_2O_2 additions to the buffer the detection range can be increased up to 1 mM with complete conversion (Fig. 2, paper II). The reactor stability depends on the enzyme batch. A purified enzyme could, however, be used for two months without serious loss of performance. The used detection method was the same as in paper I.

L-aminoacid oxidase has been used with other types of detectors, according to the principles discussed for urease and by following the decrease in dissolved oxygen or the formation of hydrogen peroxide^{4,15,17,19}. The reactivity of the different aminoacids varies, and if the sample consists of a mixture, a pre separation step has to be included (as in paper V).

The knowledge of the behaviour of enzyme reactor detection systems in plug flow can be applied to continuous measurement which is of interest for monitoring a patient status in clinical chemistry or a chemical process in industry. The precision in the measurement is higher compared to a dynamic system, especially when detectors with high noise level are used.

5.1.2 Flow Injection Analysis (FIA)

FIA

The principles of FIA have recently been described and reviewed by Růžička and Hansen⁴⁶. In this technique the sample is injected directly into a carrier stream. A strict control of the dispersion leads to reproducible results. The technique is used for sample dilution, chemical reactions in the flow, dialysis, titration, extraction and enzymatic analysis⁴⁶. Comparison between different flow methods, theoretical evaluations and applications are also given⁴⁷.

Dialysis

Dialysis is used in clinical chemistry to exclude large molecules like serum proteins or sediments from the detection system. The sample is separated from the detector by a semipermeable dialysis membrane which will exclude high molecular weight species. The dialysis is a diffusional process in which the flux of substance through the membrane is proportional to the concentration gradient over the membrane. A theoretical evaluation shows that even for high dialysis factors there is a constant relation between the concentration entering the dialyzer and the dialyzate concentration if the flow-rate, length and temperature of the dialyzer are held constant.

That this is so was shown experimentally, using catechol as a model substance. For ammonium the proportionality holds over three decades of concentration with a dialysis efficiency of 27% (paper IV, Fig. 7). Catechol was also used to quantify the

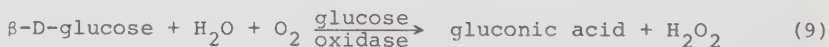
contribution to dispersion from the dialyzer, the enzyme reactor and the remaining parts of the flow system (Figs. 2 and 3, paper IV).

Urea

Urea was determined in a FIA-system with on-line dialysis and enzyme reactors. Compared to the earlier steady-state method (paper I) the range has been extended upwards due to a controlled dilution (dispersion) and due to the use of the dialyzer (Fig. 7, paper IV). Even with the long response time of the NH_3 -probe, a relatively high sample through-put was obtained (Fig. 6, paper IV). The concentration range is suitable for serum determinations. Urine samples, on the other hand, can be further diluted or a smaller dialyzer can be used.

Glucose

The number of glucose determinations in clinical laboratories is overwhelmingly large. Glucose determinations are also of interest in the food industry. Glucose oxidase (EC 1.1.3.4), a flavo-protein, catalyses the reaction,



The H_2O_2 formed is used in another enzymatic reaction catalysed by Peroxidase (EC 1.11.1.7), a protohemin IX-protein (paper IV). A strongly absorbing compound is formed. The linear calibration curves show that the system is suitable for both serum and urine samples (Fig. 5, paper IV). However, due to variations in ionic strength, further adaptations of the system has to be made for each particular type of samples.

An application of FIA without a dialysis step utilizes glucose oxidase with quinone as an oxidant in an oxygenfree stream of buffer. The reaction is followed by a potentiometric measurement of hydroquinone-quinone in the solution⁴⁸. Injection of samples in a flow-system utilizing immobilized glucose oxidase - peroxidase and spectrophotometrical measurement of the formed I_3^- have been performed. No dialysis step was used⁴⁹.

One draw-back of the described FIA-systems is that the benefits gained by the insensitivity to flow and temperature variations of the reactor detection system is partly lost in the dialysis step.

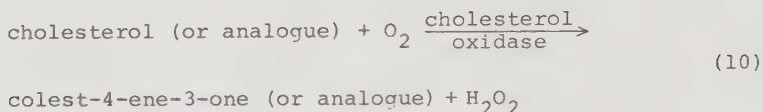
5.1.3 Post-enzyme reactors in HPLC

In the use of HPLC the detection is normally restricted to u.v.-absorption, refractive index and fluorescence measurement. Pre- or post-column derivatizations are often used to increase the sensitivity. By utilizing an enzyme reactor as a post-column component the sensitivity and specificity of the HPLC-determination can be increased.

Cholesterol and oxidized cholesterols

The analysis of cholesterol and oxidized cholesterol analogues is important, as it could help to clarify the role of these compounds in arteriosclerosis and myocardial infarction⁴. Samples with these compounds can be separated on a C-18 column with a mobile phase of ethanol-water (75+25). The detection is normally performed at 211 nm, but the performance of the detector is degraded at this wavelength.

An enzymatic reaction with cholesterol oxidase (EC 1.1.3.6.) was used (paper V),



The detection is highly specific as the enzyme only reacts with 3-hydroxy sterols (some of them are shown in Table 1, paper V). The products of the reaction absorb strongly at 241 nm. A complete conversion of the substrate is not, however, attained in the system, since an eluent with a high alcoholic content had to be used for the separation. The sensitivity is improved by both a stronger absorption of the products and a lower noise level of the detector at 241 nm compared to direct measurement at 211 nm. More sensitive measuring ranges could therefore be used. However, some of the sensitivity is lost as the effluent has to be diluted to decrease the alcohol concentration in the reactor.

5.2 Measurement of inhibitors

Mercury

Determination of mercury is of great importance in environmental control. Losses during sampling and storage^{50,51} cause the difficulties in the determination of low concentrations of mercury. Papers VI and VII describe a method for the determination of mercury. Mercury ions inhibit urease immobilized in a reactor. The sensitivity can be increased by using larger sample volumes. Mercury can be redistributed from weak to strong inhibiting binding sites

with chloride also increasing the sensitivity (paper VII). Many of the storage problems are solved by using the reactor for direct sampling. According to the theory for the measurement of inhibitors (2.2), linear calibration curves were obtained. The sensitivity of Ag^+ is the same as for Hg^{2+} and for Cu^{2+} it is 50 - 1000 times less, depending on the sampling flow-rate. The effects from other metals are small. Still only mercury gives a lasting inhibition.

Organo-mercury compounds, such as methyl- and phenyl-mercury, could also be determined with this system (Table 2, paper VII). Different reactors prepared from the same enzyme batch show equal sensitivity towards mercury (Table 1, paper VII). This system should be useful in environmental control as the strong mercury-enzyme complex eliminates mercury from a solution with $5 \cdot 10^{-18}$ M in free ions (paper VII).

A similar system has been presented by Mattiasson et al⁵² using the enzyme thermistor, but the sensitivity is lower due to the higher enzyme loading. Other inhibitor determinations have been made with immobilized enzymes. Detection of nerve-gases and pesticides in air and water with choline esterase is described by Goodson and Jacobs⁵³.

5.3 Measurement of activators

The use of enzymes to measure activators (as essential metals) is a relatively specific method. Lehky and Stein used soluble aminopeptidase for the detection of Zn^{2+} in the range $5\text{-}50 \text{ ng l}^{-1}$ ⁵⁴.

Immobilized tyrosinase was used by Mattiasson et al⁵⁵ both in an enzyme electrode and in a reactor for detection of copper down to 25 ppb. Determination of FAD, down to 10^{-12} M, with apo-glucose oxidase, has also been studied⁵⁶.

A method utilizing carboxypeptidase A (EC 3.4.12.2) has been reported⁵⁷. The enzyme shows a peptidase activity for the zinc-enzyme and an esterase activity for the cadmium- or mercury-enzyme^{58,59}. Elimination of the metal gives an apo-enzyme. The presence of zinc confers peptidase activity proportional to the added amount of zinc, see Fig. 2.

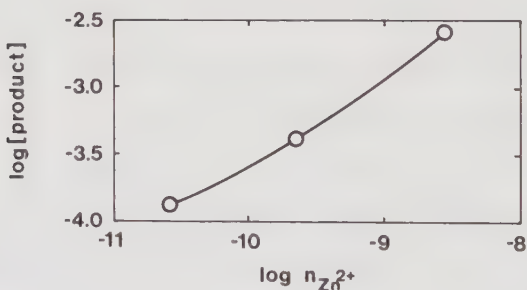


Fig. 2. Activity of an apo-carboxypeptidase A reactor as a function of amount of added zinc (moles).

The curvature in the calibration curve is dependent on zinc impurities in the buffer. Due to the two catalytic functions of the enzyme it might be possible to measure both zinc and the sum of cadmium and mercury simultaneously.

6. CONCLUSIONS

The use of enzyme reactors has many advantages over soluble enzymes and enzyme electrodes. Complete conversion over a large concentration range and few restrictions in the choice of detector, due to the separation of the enzymatic reaction and the detector, are the most important ones. The theory for the enzyme reactor can be used as a guide in the construction of an analytical system. In FIA, with on-line dialysis, optimization to a special analytical application can give low dead volume and high throughput rate for the samples. Using the enzyme reactor as an enzyme kinetic amplifier, with inhibitors and activators steering the enzymatic activity, one obtains high sensitivity and selective analytical methods for these compounds.

Development of highly sensitive detection systems, such as chemiluminescence⁶⁰ have decreased the detection limit. One fifth of all enzymes are NAD^+ - or NADP^+ -dependent. Techniques for coimmobilization of enzymes and coenzymes⁶¹, for electrochemical regeneration⁶² and for electrochemical measurements⁶³ have already started to open up a new field of applications which will certainly be much more important in the future.

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AN ENZYME REACTOR ELECTRODE FOR UREA DETERMINATIONS

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SUMMARY

An enzyme reactor electrode system for the determination of urea is described. A buffer is pumped through an enzyme reactor (0.4 ml) containing urease immobilized with glutaraldehyde to glass. The effluent is mixed with sodium hydroxide pumped through a second channel and fed through an ammonia gas electrode. Samples are introduced via a third flow channel and mixed with the buffer. The conversion of urea to ammonia is quantitative for sample concentrations of less than 0.03 M for a flow rate of 40 ml h⁻¹. The reactor electrode shows a Nernstian slope of 57 mV/decade for $5 \cdot 10^{-5}$ – $3 \cdot 10^{-2}$ M urea. The response is independent of variations in the flow rate, enzyme activity or temperature of the reactor.

During recent years considerable interest has been focused on enzyme electrodes. Much of the work reported on successive improvements of the technique has been based on the determination of urea as an example. At first, urease was immobilized in a gelatinous layer wrapped round a cation-selective glass electrode [1–3]; the electrode responded to the ammonium ions produced in the enzymatic reaction. The selectivity of the glass electrode was not very high and therefore other approaches were studied. The nonactin electrode [4] provided better selectivity for ammonium over the alkali metals [5]. Further refinements reduced the interferences [6] to a level where blood serum measurements became feasible, despite some remaining interferences [7, 8]. Other approaches for urea determinations have included sensing of the pH-changes of a polyacrylamide gel containing entrapped urease and wrapped around a pH-electrode [9]. The carbon dioxide level has also been monitored [10] and ion exchangers have been used to improve the selectivity [11]. The ammonia gas electrode [12] can provide an unlimited selectivity for ammonia over ions in the solution. This electrode has been covered with a layer of enzyme on the surface [13] and used for urea determinations. It has also been used in a flow system containing soluble urease [14]. The air-gap electrode [15] also provides excellent selectivity [16] and has been used for urea determinations in blood and serum [17, 18]. A metal sulphide gas electrode can also be used for ammonia determinations above $10^{-3.5}$ M ammonia [19]. A double-membrane urea electrode was recently described [20]. Several reviews have summed up the work on enzyme electrodes [21–23].

Substantial improvements have resulted from the work mentioned above but still several problems remain to be solved, the most notable being the variation of the signal with stirring and the change of the calibration with time. In the air-gap electrodes [16–18], the enzyme is separated by the air-gap from the sensing electrode surface, and this allows the enzyme reaction and the release of ammonia to be conducted at their optimal pH values [16].

In the present paper, a rather different approach is described. The enzyme is separated entirely from the electrode so that both the enzyme and the electrode are operated at their optimum pH. The immobilized enzyme is contained in a small reactor in a flow system operated under such conditions that the substrate is converted to products with 100 % efficiency. The effluent from the reactor is made alkaline with a flow of sodium hydroxide and passed into a flow-through gas electrode.

An enzyme reactor has been used analytically in connection with flow microcalorimetry [24] as well as on a larger scale where fractions of the effluent were analyzed with a cation-selective electrode [25]. An arrangement utilizing similar ideas as the reactor electrode has been described by Sundaram [26], who immobilized urease on the inside of a plastic tubing.

EXPERIMENTAL

Apparatus

The flow system shown in Fig. 1 was used for the measurements. A three-channel peristaltic pump or an infusion pump with three all-glass syringes was used. The buffer was 0.2 M Tris-HCl (pH 7.0) and 2mM EDTA. The next channel contained water which could either flow directly to a mixer or be diverted through a sample loop. The samples were thus interspaced with water. The buffered sample was fed into the enzyme reactor and then mixed with 1.4 M sodium hydroxide. The effluent was passed through a heat-exchanger coil (650 mm long, i.d. 0.5 mm, volume 140 μ l) which was immersed in water. The effluent was then passed through the flow chamber of an EIL ammonia probe (Electronic Instruments Ltd., England, Model 8002-2). This gas electrode for ammonia contains a membrane permeable

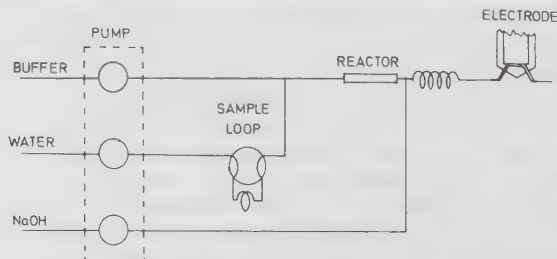


Fig. 1. Flow system for the enzyme reactor electrode.

to ammonia but impermeable for ions; its performance was recently evaluated [27]. The potential of the glass electrode in the ammonia probe was monitored by a pH-meter provided with a strip chart recorder and a printer in parallel. The tubings, connectors, heat exchangers and the sample loop were assembled from teflon components for liquid chromatography (Altex).

Two enzyme reactors, A and B, were used. Reactor A was 35 mm long, (i.d. 6 mm, volume 1 ml) and contained polyacrylamide gel with immobilized urease (Sigma Chemical Co., U-5376). Reactor B was 45 mm long (i.d. 3.4 mm, volume 400 μ l) and contained urease immobilized on glass beads (see below). Filter discs were inserted at the ends and the connections were made so that the dead volume was very small.

Immobilization of urease

The urease was covalently bound to glass via glutaraldehyde coupling, by methods pioneered by Weetall and Hersch [25]. This is one of the simplest and gentlest coupling methods [28]. The technique has also been used with the enzyme thermistor [29].

CPG-10 controlled pore glass (10 ml; diameter 70–150 μ m, pore diameter 70 nm; Corning Glass Works, N.Y.) was boiled in 150 ml of 5 % nitric acid for 45 min. The glass beads were washed with about 1 l of water on a glass filter G3 and dried in an oven at 95 °C.

3-Aminopropyltriethoxysilane (5 g) was added to 45 ml of water and the pH was carefully adjusted to 3.45 with 5 M HCl. The dried glass was added, the pH checked and then the mixture was kept at 75 °C on a water bath for 2 h 45 min. The flask was swirled every 15 min. The mixture was filtered, washed and dried as described above. The alkylamino glass can be stored in this condition until batches are needed.

A buffer containing 0.1 M sodium phosphate (pH 7.0) and 2.5 % glutaraldehyde was prepared by adding the aldehyde (BDH Chemicals) from a 25 % stock solution just before use. Alkylamino glass (1 g) was added to 5 g of the glutaraldehyde buffer. The reaction was allowed to proceed for 1 h at room temperature, the first 30 min at reduced pressure to decrease the oxygen partial pressure. The activated glass was washed well with distilled water.

Urease (100 mg; Sigma U-1500, 1500–3000 U g⁻¹) was dissolved in 3 ml of cold (+4 °C) 0.1 M sodium phosphate (pH 6.0) and the activated glass was added. The solution was kept at +4 °C for 2.5 h, the first 30 min at reduced pressure. The glass beads were washed with 250 ml of cold buffer and 500 ml of cold water and stored moist at +4 °C.

RESULTS

The ammonia probe was calibrated with ammonium chloride solutions introduced via the sample loop, the enzyme reactor being removed during this operation. The response was strictly linear between 0.2 and 10⁻⁵ M NH₄⁺

in the sample. The slope was 57.3 mV/decade, compared to a Nernstian value of 58.4 mV/decade.

Next the column with polyacrylamide immobilized enzyme, reactor A, was inserted and samples of urea in water were passed through. For a total flow rate of 22 ml h⁻¹ a linear response was obtained between 10⁻² and 10⁻⁴ M urea in the samples. For a total flow rate of 7.8 ml h⁻¹, the upper limit of linearity was extended to 5 · 10⁻² M urea. If a slightly curved calibration curve was acceptable the arrangement could be used for measurements down to 10⁻⁵ M urea. The gel could not be tightly packed as then the flow would be hindered. Therefore a loose suspension, 60 mg in 1 ml, had to be used and the reactor thus had a considerable dead volume. At lower flow rate, 10–35 min were required before a steady-state was attained.

The column containing urease immobilized on glass, reactor B, proved to be much more efficient and to have a low dead volume. A calibration curve is shown in Fig. 2; it can be seen that for a flow rate of 40 ml h⁻¹ the response is linear between 5 · 10⁻⁵ and 3 · 10⁻² M urea with a slope of 56.9 mV/decade. The urea is thus converted to ammonia with 100 % efficiency over the linear range. Figure 3 shows the recordings from successive samples of urea; the samples were introduced before the base line had been reached. In fact there were some pockets in the flow system so that the time to return to the base line after a concentrated sample was rather long. If the samples have about the same concentration equilibrium is reached within 2.5–3.5 min. As can be seen in Fig. 3 there will be a flat portion and the length of this depends

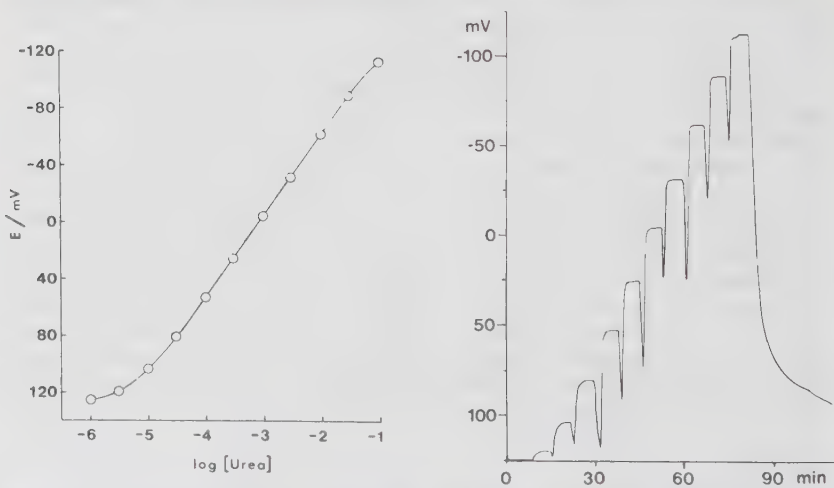


Fig. 2. Response curve for the enzyme reactor electrode with reactor B (urea immobilized to glass). Concentration of urea relates to the sample concentrations. Flow rate 40 ml h⁻¹.

Fig. 3. Recorder trace for 1.9-ml samples of 3 · 10⁻⁶, 10⁻⁵, 3 · 10⁻⁵, 10⁻⁴, 3 · 10⁻⁴, 10⁻³, 3 · 10⁻³, 10⁻², 3 · 10⁻² and 10⁻¹ M urea.

on the sample size; 1.9-ml samples were used for the recordings shown in Fig. 3. Under these conditions up to 8 samples could be run per hour. The response time is limited by the ammonia probe, because it takes some time before a diffusion equilibrium is set up over the membrane. To indicate the overall stability, the voltage recordings printed every 30 s are shown in Table 1; it can be seen that the potential is extremely stable except for the highest concentration where the conversion efficiency is less than 100 %.

Eleven samples containing accurately known urea concentrations evenly spaced between 10^{-3} and 10^{-2} M were analyzed. A line was calculated with a least-squares procedure and the standard deviation amounted to 0.23 mV at 40 ml h⁻¹ and 0.15 mV at 23 ml h⁻¹. The results are thus better than the stated reproducibility of the ammonia sensor, 2 %.

A diluted urine sample was analyzed with the enzyme reactor electrode. A direct determination gave a value of 2.08 mM urea and with standard addition a value of 2.09 mM urea.

The two most important sources of error are related to the operation of the ammonia sensor. Variations of the temperature of the flowing sample cause uneven temperature distribution within the probe. Differences in temperature between an external reference electrode and the reference electrode inside a glass electrode are known to produce very large e.m.f. errors. The time constant for thermal equilibrium is of the order of 30 min. In the flow system described, heat will be evolved by the enzymatic reaction and during the addition of sodium hydroxide. To reduce the temperature fluctuations a thermal buffer consisting of a coil immersed in a beaker with water was added. The other source of error for the sensor is the disturbance caused by gas bubbles in the flow chamber. As the solubility of air in sodium hydroxide is less than in water, the solution may be super-saturated with oxygen after the mixing "T". The heat exchanger had a beneficial effect as it also cooled the flowing solution. In some cases the buffer was also deaerated somewhat.

Exactly the same calibration curve was obtained at a flow rate of 23 ml h⁻¹ as at 40 ml h⁻¹, except that the range was increased somewhat at the upper end. An enzyme column only 22 mm long, thus containing only half as much enzyme, was also used and gave identical results except that the upper limit

TABLE 1

Voltage recordings in mV of the urea measuring system as a function of time and concentration

Urea concn.	Time(min)					
	2.5	3.0	3.5	4.0	4.5	5.0
$3 \cdot 10^{-6}$	120.0	119.5	119.4	119.4	119.4	119.5
$1 \cdot 10^{-4}$	53.4	53.2	53.1	53.1	53.1	53.4
$1 \cdot 10^{-3}$	-3.3	-3.5	-3.5	-3.5	-3.5	-3.6
$1 \cdot 10^{-2}$	-60.5	-60.5	-60.5	-60.4	-60.3	-60.4
$1 \cdot 10^{-1}$	-109.0	-109.6	-109.9	-109.8	-110.0	-110.2

for linear response decreased somewhat. The enzyme columns were stored in a refrigerator during the night and could be used for about one month without any appreciable loss of activity. Heavy metals are known to inhibit the enzyme, but it was found that if samples containing 1 mM lead(II) or 1 mM cadmium(II) were run through the activity returned to normal as fast as the metal ions were washed away with the buffer.

Ammonium ions in the solution can be differentiated from urea by inserting a column of ion exchanger. A column (70 mm long, i.d. 2 mm) was filled with Dowex AG50W-X8, (100–200 mesh, sodium form) and inserted in the flow system after the sample loop and before the mixing T-tube (Fig. 1). When samples containing up to 10^{-2} M ammonium chloride were introduced, the ammonium ions were completely removed. The capacity of the small column was estimated to be sufficient for removal of the ammonium ions from several hundred serum or urine samples. The ion exchanger is sensitive to increases in the electrolyte concentration, so that 1 M NaCl elutes the ammonium ions. When samples of urea were also run through the system with the ion-exchange column in place, no absorption of urea occurred. In clinical chemistry, ion exchangers for removal of ammonium ions have been suspected of urea absorption. The results presented here show that this is not the case for aqueous solutions of urea.

DISCUSSION

The enzyme reactor electrode operates with complete conversion of the substrate to the required products. Over the linear range, there is excess of enzyme activity and variations in flow rate, in the temperature of the reactor or in the enzyme activity do not affect the response. Most earlier types of enzyme electrode depend on a diffusion equilibrium over a thin layer. The diffusion rate depends on temperature and the diffusion layer is affected by stirring. The enzyme reactor electrode, however, gives a response curve with the same slopes as that of the glass electrode. The reactor electrode can be calibrated with ammonium chloride solutions as well as with urea solutions. Another advantage of the enzyme reactor electrode concept is that both the enzyme reactor and the ammonia probe can be operated at their optimum pH values. This is in contrast to covered enzyme electrodes where a compromise is necessary; changes in pH may then result in errors because of variations in the $\text{NH}_3/\text{NH}_4^+$ -ratio.

The working conditions can be strictly controlled for the enzyme reactor electrode so that a very precise result can be obtained. The weakest part of the system is the ammonia sensor, which has a rather long time constant. The calibration curve is displaced when the ionic strength of the solution changes, probably because of variations in the activity coefficient of ammonia in the flowing solution.

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AN ENZYME REACTOR ELECTRODE FOR DETERMINATION OF AMINO ACIDS

GILLIS JOHANSSON, KERSTIN EDSTRÖM and LARS ÖGREN

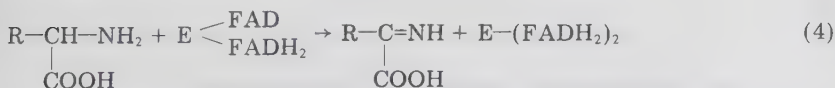
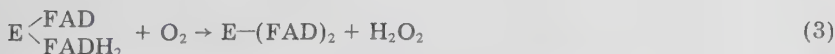
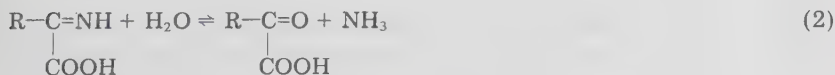
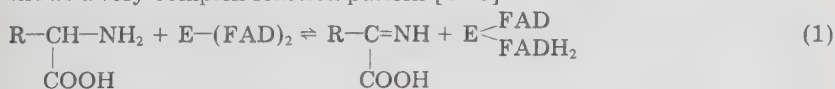
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(Received 11th February 1976)

SUMMARY

L-Leucine can be determined with an enzyme reactor electrode containing L-amino acid oxidase immobilized with glutaraldehyde to glass. The reactor also contains immobilized catalase which splits the hydrogen peroxide formed. Oxygen for the reaction is also supplied by adding hydrogen peroxide to the samples. The electrode is an ammonia gas sensor. The calibration curve is strictly linear with Nernstian slope between $3 \cdot 10^{-5}$ and 10^{-3} M leucine.

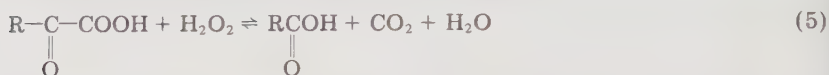
It was recently shown [1] that an enzyme reactor electrode for urea has various advantages over other types of enzyme electrodes. Urease is known to have a very high turnover rate and the kinetics are relatively simple. In order to test the reactor electrode concept further, an enzyme reaction with complicated kinetics and dependence on two substrates was selected. L-Amino acid oxidase has been used earlier in enzyme electrodes [2–5], and shows a very complex reaction pattern [6–8]



The enzyme contains two moles of flavin adenine dinucleotide (FAD) per mole of enzyme. The first is readily reduced (eqn. 1) and reoxidized (eqn. 3). The second FAD molecule can also be reduced in the presence of excess of substrate (eqn. 4), but its reoxidation is slow. Consequently, the enzyme is severely inhibited by excess of substrate. If the normal reoxidation is retarded

by oxygen deficiency, conversion to the fully reduced enzyme complex will be favoured. High substrate concentrations as well as oxygen deficiency are therefore expected to be detrimental to the operation of an enzyme reactor column.

Three methods have been used to follow the enzymatic reaction: measuring the consumption of oxygen, the production of ammonia or the production of hydrogen peroxide. Monitoring the hydrogen peroxide has some drawbacks, as it may react with the α -ketoacid in a side-reaction.



The amount of oxygen available can be increased by using catalase



Oxidation of one mole of amino acid requires one mole of oxygen. If immobilized catalase is added to the column, half a mole of oxygen per mole of amino acid can be recycled.

EXPERIMENTAL

Enzyme immobilization

Purified L-amino acid oxidase from snake venom (Sigma Chemical Co., A 9378, 3–6 units/mg) was immobilized on CPG-10 controlled-pore glass (Corning Glass, pore diam. 70 nm, 120–200 mesh) as described earlier [1]. Enzyme (5 mg) in 3 ml of buffer was coupled with glutaraldehyde to 0.5 g of alkylamino-activated glass.

Catalase (5 mg; Sigma Chemical Co., C-40, 10000–25000 Sigma units/mg) was immobilized to 0.5 g of activated glass by the same procedure.

Flow system (Fig. 1)

A three-channel peristaltic pump (Pharmacia Fine Chemicals, Model P3) was used with 2.1- and 1.0-mm tubing, so that the flow could be varied over the ranges 2–32 ml h⁻¹ or 0.6–9 ml h⁻¹ per channel. The buffer was 0.05 M sodium phosphate containing 1 mM EDTA (pH 7.0). The enzyme reactor consisted of a PVC tube (i.d. 3.2 mm, length 45 mm) threaded at the ends to fit Altex or Chromatronix couplings for teflon tubes. The enzyme-coated glass beads were tightly packed into the column and silver frits (1/8 in. diameter with 0.015-mm holes; Reeve Angel, Cat. No. LA 230) were fitted at the ends. The connecting tubing and the heat exchanger were made of teflon (i.d. 0.5 mm; Altex Scientific, Berkeley).

The heat exchanger and the ammonia flow-through electrode (E.I.L. ammonia probe, Model 8002) were immersed in a bath thermostated at 25.0 °C. The enzyme reactor was immersed in a bath which was kept at 37 ± 1 °C in most of the experiments. The electrode was connected to a digital pH meter provided with a strip chart recorder.

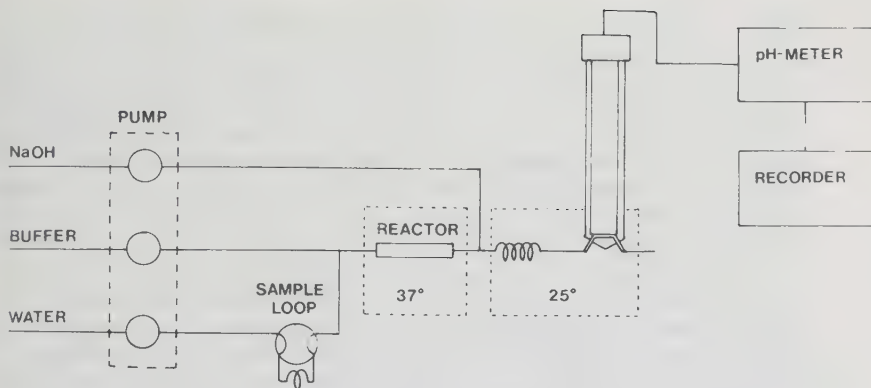


Fig. 1. Diagram of the flow system and the enzyme reactor electrode arrangement.

Samples were introduced via a sampling valve (Altex 4-way Rotary Valve, series 202) provided with a sample loop of about 2.3 ml.

Operation

A sample of amino acid was injected into the sample loop in the bypass position (see Fig. 1). In this position water was mixed with the buffer and the diluted buffer passed through the enzyme reactor to the T-joint, where it was mixed with 0.5 M sodium hydroxide solution. The alkaline solution then passed through the ammonia probe, giving a base-line on the recorder (corresponding to less than 10^{-6} M ammonia in the sample). When the flow was switched to pass through the sample loop, the sample was mixed with buffer and passed into the enzyme reactor. Under optimal conditions, an equivalent amount of ammonia was split off and measured. With samples of adequate size a steady state was attained, so that the ammonia concentration in the flow-through electrode was constant for a short time. The ammonia probe then attained equilibrium and the recorder trace was flat at the new level. The digital reading on the pH meter was noted, as it was more accurate than the recorder. When the sample had been eluted, it was followed by water, and the recorder then returned to the base-line.

RESULTS AND DISCUSSION

Tests were made with the purified as well as the crude enzyme from the venom of *Crotalus adamanteus*. The preparations made from the crude enzyme lost activity within a couple of days, whereas preparations from the purified enzyme retained adequate activity for more than two months. As there might have been some loss of FAD, an attempt was made to rejuvenate the column with FAD after a period of use, but no increase in activity could

be seen. The experiments reported below were all made with the purified enzyme.

Oxygen for reaction (3) is supplied from oxygen dissolved in the sample and the buffer. This puts an upper limit on the concentration of amino acid which can be oxidized. At equal flow rates in the sample and buffer channels the upper limit was calculated to be $5 \cdot 10^{-4}$ M amino acid, and this was confirmed by experiment. By utilizing catalase it should be possible to extend the range to about $8 \cdot 10^{-4}$ M.

The immobilized catalase was first filled into a column and tested at room temperature. Samples of hydrogen peroxide mixed with buffer were run through the column, and the effluent was collected and analyzed spectrophotometrically at 240 nm; the amount of unreacted hydrogen peroxide was evaluated from a calibration curve. It was found that $7 \cdot 10^{-4}$ M hydrogen peroxide remained unreacted if 0.1 M hydrogen peroxide was pumped into the reactor at a flow rate of 21 ml h^{-1} . If 0.02 M hydrogen peroxide was run through the reactor, the absorption of the effluent was equal to the blank, i.e. less than 10^{-4} M hydrogen peroxide. The immobilized catalase was thus a very efficient catalyst for eqn. (6).

Immobilized L-amino acid oxidase and immobilized catalase were mixed in the proportion 3:1 and filled into the reactor. This reactor, operated at $37 \pm 1^\circ \text{C}$, was used in all experiments described below. The pH-optimum is known to depend on the substrate [7], and the range is quite wide for an amino acid such as L-leucine. Runs were made at pH 7.0, 7.6 and 8.0 which confirmed that the activity of the immobilized enzyme was practically independent of pH for L-leucine.

Table 1 shows the results obtained with samples of 1 mM L-leucine. Using an air-saturated buffer the conversion was found to be incomplete at the tested flow rates. The reason is that the oxygen concentration is so low that the enzyme becomes fully reduced, with a resulting decrease in its turnover rate. If oxygen is bubbled through the buffer, the efficiency of the reactor increases somewhat, but it is still low and depends on the flow rate. The

TABLE 1

Voltage reading of the enzyme reactor electrode when air or oxygen was flushed through the buffer

Sample	Concn. (mM)	Flow rate (ml h ⁻¹)	Gas	Reading (mV)
NH ₄ Cl	1	21	air	-2.0
L-Leucine	1	21	air	4.0
L-Leucine	1	13.5	air	0.3
L-Leucine	1	21	O ₂	0.9
L-Leucine	1	13.5	O ₂	-0.6
L-Leucine	0.1	21	O ₂	57.5
L-Leucine	0.01	21	O ₂	114.2

electrode response is 4 % lower than in the ammonia standard. Lower concentrations were also used; Table 1 shows that the response is almost Nernstian, being 58.1 mV/decade to 0.1 mM and 56.7 mV/decade between 0.1 and 0.01 mM leucine.

It is possible to increase the amount of oxygen by adding hydrogen peroxide to the sample or the buffer. If the hydrogen peroxide is added to the buffer, there will be an excess of oxygen in the absence of substrate. It was found that at most $2 \cdot 10^{-3}$ M hydrogen peroxide could be added before oxygen bubbles in the flow caused malfunction of the ammonia sensor. Hydrogen peroxide was therefore added to the samples.

A calibration curve for L-leucine was run at a flow rate of 13.5 ml h^{-1} , see Fig. 2. The samples contained hydrogen peroxide in the same concentration as the amino acid. It can be seen that the enzyme reactor electrode operates ideally between $3 \cdot 10^{-5}$ and 10^{-3} M leucine with an almost Nernstian slope, 57.7 mV/decade. The reproducibility in this range is better than 1 %. Ammonium standards are also shown, and the agreement between standard and sample is excellent at 10^{-3} and 10^{-4} M. At 10^{-5} M the rate of reaction is too low for complete conversion. As the hydrogen peroxide was added at the same concentration as the leucine the increase in oxygen pressure is very small at this concentration; a higher concentration of peroxide may increase the rate. At concentrations higher than 10^{-3} M substrate, inhibition occurs in the first part of the reactor, decreasing the overall efficiency. There are deviations from the straight line at $3 \cdot 10^{-3}$ M

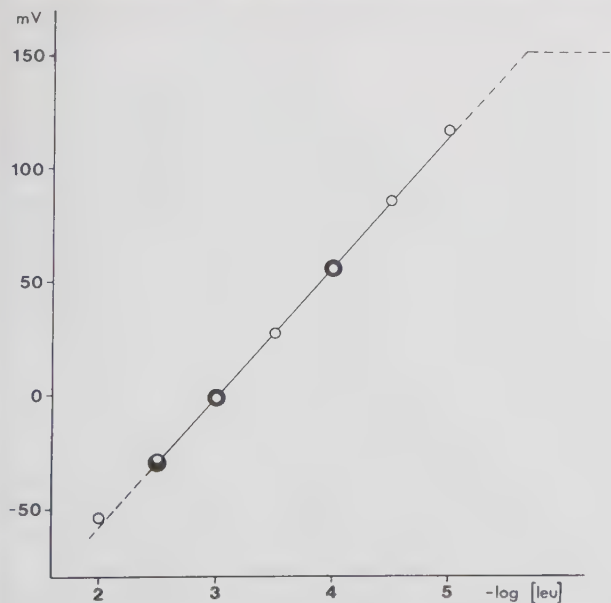


Fig. 2. Calibration curve for L-leucine($\circ$) and for NH_4Cl standards ($\bullet$).

leucine and higher. The results shown in Fig. 2 were obtained with an enzyme reactor 2.5-months old, the activity of which had decreased substantially. New columns give better performance.

The results presented above show that the enzyme reactor electrode can be used with enzymes which have a complex kinetic pattern. They also prove that by adding catalase and hydrogen peroxide an oxygen-dependent system can be used in the reactor. The device is linear in the concentration range of interest and the precision is high. When the flow is decreased sufficiently, the response is independent of flow fluctuations.

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APPLICATION OF ION-SELECTIVE ELECTRODES TO ENZYMATIC ANALYSIS

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ABSTRACT

An enzyme reactor electrode for urease was studied and its kinetic behavior is reported. The parameters, K_m and v_{max} , necessary for the design of a reactor operating with $\square\%$ conversion efficiency, were evaluated. The design of reactors with two cofactor dependent enzymes is discussed. By proper selection of enzyme systems it should be possible to find new applications in which the ammonia probe can be used as a sensor. Some preliminary results for l-alanine-dehydrogenase are reported.

INTRODUCTION

The combination of an enzyme reaction with an ion-selective electrode opens up a new field of analytical applications. The enzymes give a high selectivity to the compounded system. Further measurements can be made on substances which are unionized and thus otherwise out of reach of the potentiometric methods. The rapid progress in the development of enzyme electrodes have been described in a number of excellent reviews and therefore I will concentrate only on the enzyme reactor concept.

Enzyme reactors have been used for some time in enzyme engineering¹⁻². The rapid development of immobilization technique has resulted in much more efficient reactors as well as much more stable enzyme preparations.

EXPERIMENTAL

Enzymes were immobilized on porous glass as described previously³⁻⁴ (Cornig Glass, pore diam 70 nm, 120-240 mesh) using glutaraldehyde as a coupling reagent. The immobilized glass was filled into reactors 3.2 or 2.0 mm i.d. treaded at the ends to accommodate Altex or Chromatronix couplings for teflon tubes. Silver frits, 1/8" diam, with 0.015 mm holes were fitted at the reactor ends.

The reactor was inserted into a flow system as shown in Fig 1. The sample was introduced via a sample loop and mixed

with a buffer. The buffered sample was fed into the enzyme reactor where the reaction was allowed to go to completion. The figure shows the arrangement for ammonia monitoring with the ammonia gas electrode (EIL, Model 8002). A stream of NaOH was mixed with the reactor effluent in order to displace the ammonia protolysis.

The calibration was made with NH_4Cl standard solutions introduced via the sample loop. The ammonia concentrations given below thus refer to those of the sample and not to those actually passing the electrode. There is a dilution before the solution enters the electrode. In order to improve the electrode accuracy it was kept in a thermostat at 25°C . The flow also passed a coil of teflon tubing immersed in the thermostat before entering the flow-through electrode.

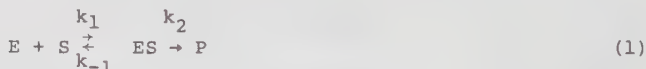
RESULTS AND DISCUSSION

Urease determination

Urease (Sigma U-1500, $1500-3000 \text{ U g}^{-1}$) was immobilized to glass and filled into a 45 mm long reactor. Samples of urea were analyzed and Fig 2 shows the result for a flow rate of 40 ml h^{-1} . The urea is converted to ammonia with 100% efficiency in the range $5 \cdot 10^{-5}$ to $3 \cdot 10^{-2} \text{ M}$, the calibration curve is strictly linear. At higher concentrations the conversion seems to be incomplete.

The kinetics of an urease reactor

The kinetics of enzyme reactors have been studied by several workers, see e.g. Goldstein and Katcholski⁵. Suppose that we have a simple enzyme reacting, an enzyme (E) reacts with a substrate (S) giving the product (P).



If the reactor has the area A and the flow is $q \text{ ml/min}$, it is easy to calculate the product concentration p at some distance x along the reactor, see Fig 3. The substrate concentration is S_0 before reaction starts. Using Michaelis-Mentens equation the following expression is obtained

$$\frac{dp}{dt} = \frac{v_{\max} (S_0 - p)}{K_S + (S_0 - p)} \quad (2)$$

with

$$K_S \equiv (k_{-1} + k_2)/k_1$$

The subscript zero indicates concentrations at the inlet and outlet of the reactor. This equation can be integrated over the reactor length and the time can be replaced with symbols for area and flow rate. The following equation is obtained

$$\frac{v_{\max}}{K_S} \cdot \frac{A}{q} x = p + K_S \ln \frac{S_0}{(S_0 - p)} \quad (3) \quad \propto 1$$

What length of the reactor, l , is necessary to give a conversion efficiency of $\eta = \frac{p_0}{S_0}$

If $\eta = 0.999$, i.e. the conversion is complete to within 0.1% the length should be

$$l = \frac{(S_0 - K_S \ln(1-\eta))q}{v_{\max} \cdot A} = \frac{(S_0 - 7K_S)}{v_{\max} \cdot A} \cdot q \quad (4) \quad \phi - \phi +$$

A conversion efficiency of 99% gives $-\ln(1-\eta) = 4.6$.

Both $v_{\max}$ and K_S can be measured if $S_0 \eta$ is plotted against $-\ln(1-\eta)$ for each value of the flow rate for a given column. The slope of the straight line is $-K_S$, the intercept with the ordinate $v_{\max} \frac{A}{q} \cdot l$ and with the abscissa $v_{\max} \frac{A}{q} \frac{1}{K_S}$.

η can easily be measured if alternate samples of NH_4Cl with the concentration $2S_0$ and urea samples with the concentration S_0 are run through the system and the potential difference of the electrode, ΔE , is noted.

$$\frac{p_0}{S_0} = \eta = 10^{\Delta E/L} \quad (5)$$

L is the slope of the electrode.

Fig 4 shows a plot of some kinetic data. Due to the very high efficiency of the enzyme it was necessary to use high urea concentrations and high flows as well as small columns in order to get a measurable ΔE . The back pressure was in fact so large that the pump worked somewhat erratically at the higher flow rates.

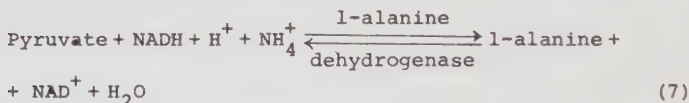
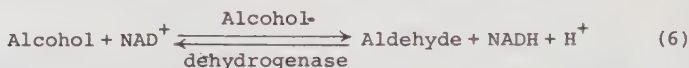
There might also be some channelling due to the difficulties in packing such a small reactor, the silver filters at the ends were far from ideal. The evaluation resulted in the following constants (25°C, pH 7.0, tris buffer).

q ml/min	0.44	0.65
K_S mM	3.1	4.3
$v_{\max}$ μ moles/min	34	43

K_S for soluble urease in phosphate buffer (25°C, pH 7.0) has been reported⁶ to be 19 mM. The apparent increase in rate with flow rate is due to experimental errors. In fact the rate should decrease with flow rate^{5,7} as the diffusion into the glass spheres may not be fast enough.

Field of application

The ammonia electrode is a very good sensor for enzyme reactions and as shown by the urease-reactor-electrode³ very good results can be obtained. The same system has also been applied to amino-acid oxidase⁴ which requires oxygen. Only a small fraction of all enzymatically catalyzed reactions produces ammonia, however. Therefore the possibilities to extend the range of this sensor by using two immobilized enzymes have been studied. A large group of enzyme is cofactor dependent. A combination of two cofactor dependent reactions, one of them involving ammonia, should be possible and should result in new applications. Consider the following two reactions



+



The equilibrium constant of eq (8) can be calculated from the known constants of eq (6) ($K = 8 \cdot 10^{-12} \text{M}$)⁸ and of eq (7) ($K = 3.1 \cdot 10^{-14}$)⁹ to be

$$\frac{[\text{Acetaldehyde}][\text{l-alanine}]}{[\text{Ethanol}][\text{Pyruvate}][\text{NH}_4^+]} = 250 \quad (9)$$

By suitable adjustment of concentrations it should thus be possible to set up an enzyme reactor electrode for ethanol determination. pH is an important parameter in the reaction as l-alanine has a pK of 9.8 and NH_3 a pK of 9.3. By selecting a pH of about 9.0 the reaction is forced towards the right eq (7) if a pH of about 10.0 eq (7) is forced to the left. A straightforward attempt to mix immobilized alcohol-dehydrogenase and l-alanine-dehydrogenase in a reactor was unsuccessful. The conversion was found to be less reproducible and flow dependent. It is therefore necessary to evaluate the kinetic parameters of the two enzyme systems in order to make an efficient reactor.

Immobilized l-alanine-dehydrogenase was filled into a reactor and the expected pH-dependence was observed. The equilibrium of eq (7) is not displaced entirely in any direction and

therefore only a partial consumption of NH_4^+ occurs even when pyruvate and NADH are in excess. The kinetic equation will therefore become more involved than for the urease reactor. It was observed, however, that the reaction was sufficiently fast for the flow system used.

Immobilized alcohol-dehydrogenase (from yeast) was filled into another reactor and the reaction eq (6) was followed spectrally at 340 nm. A test was made at pH 8.9 in a phosphate buffer with a NAD^+ -concentration of 1 mM and an ethanol concentration of 0.8 mM. It was found that the reaction was slow and showed a small flow dependence, see Fig 5.

A detailed kinetic evaluation of this reactor is necessary and it will be more complicated than for the urease reactor as NADH can inhibit the enzyme. A check of the equilibrium conditions should also be made. Presently variations in the immobilization technique are tested in order to get an immobilized enzyme with high activity.

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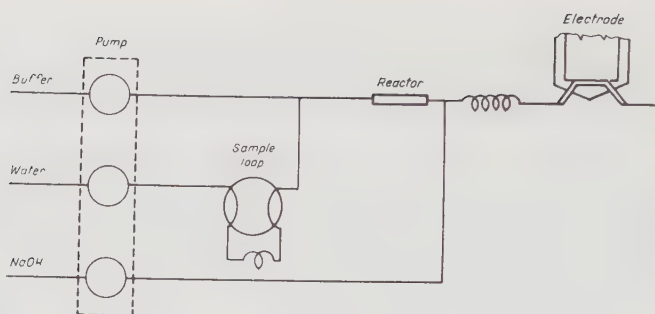


Fig. 1

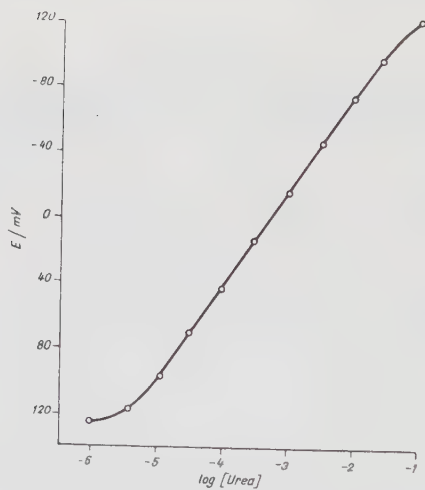


Fig. 2

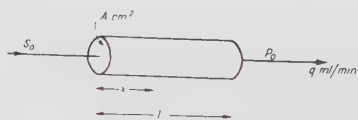


Fig. 3

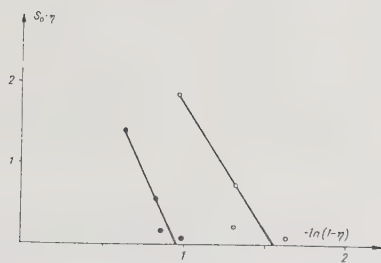


Fig. 4

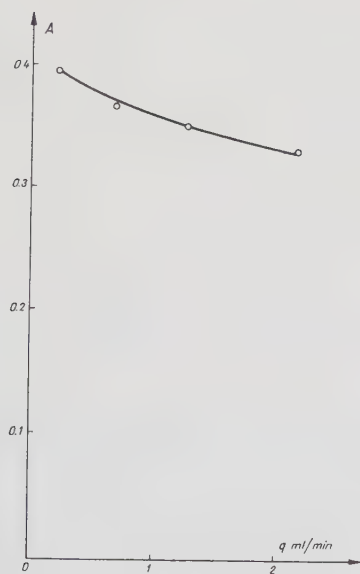


Fig. 5

QUESTIONS AND COMMENTS

Participants of the discussion: Prof. G. Johansson,
Prof. E. Pungor, Prof. J.D.R. Thomas

Questions:

1. A question concerning the stability of the enzymes: At what temperature are the enzymes usually preserved?

Answer:

The enzymes are usually stored at $+4^{\circ}\text{C}$ when not used, overnight or over weekends. The purity of the enzyme greatly affects its stability. A very careful purification can increase the life-time by a factor of 6. In favourable cases the enzymes can be stored even at 60°C without rapid loss of activity.

2. A question about the memory effects in the determination of mercury on the basis of the inhibition of an enzyme: Are serial determinations influenced by memory effects?

Answer:

We have a given amount of enzyme in the reactor, then we pass through a given volume of sample solution containing mercury. All the mercury in that volume is bound by the enzyme. After measuring the reduction in enzyme activity, the enzyme is regenerated using e.g. thioacetamide. And the reactor is ready for a new determination and no memory effect occurs. It is interesting that the enzyme can be inhibited and regenerated several hundred times without remarkable loss of activity.

FLOW INJECTION ANALYSIS OF GLUCOSE AND UREA USING
ENZYME REACTORS AND ON-LINE DIALYSIS

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Abstract

A Flow Injection Analysis (FIA) system for glucose and urea determination is described. The glucose determination utilizes immobilized glucose oxidase in a reactor designed to give 100% substrate conversion. The hydrogen peroxide formed is converted to a coloured complex using 4-aminophenazone and N,N-dimethylaniline as coreactants. The coupling is catalyzed by a reactor containing immobilized peroxidase. The coloured complex is measured in a flow-through spectrophotometric cell. Urea is split into ammonia in a reactor with immobilized urease and detected with an ammonia gas membrane electrode. Proteins and other interferants from serum samples were removed in an on-line dialyzer.

Linear calibration curves were obtained for glucose in the range $1.6 \cdot 10^{-4}$ - $1.6 \cdot 10^{-2}$ M and for urea in the range 10^{-4} - 10^{-1} M. The samples were 25 μ l for glucose determination and 100 μ l for urea determination. The range of the method can be changed by varying the sample sizes. The effects of the dialyzer, the enzyme reactors and the detectors on the dispersion are evaluated.

INTRODUCTION

Continuous flow-injection analysis (FIA) has a number of advantages for enzymatic analysis of substrates as shown by Ružička, Hansen and their coworkers.¹⁻⁴ Further advantages should be obtained if the system had been adapted to immobilized enzymes. Some on-line sample pretreatment can also be made in the system. A flow-through dialyzer should be able to replace the protein precipitation step. In practice some difficulties have been reported with the dialysis technique.^{1,5}

Enzyme reactors have been used before with the sample injection technique for the determination of glucose,⁶⁻⁷ testosterone,⁷ urea⁸⁻⁹ and uric acid.¹⁰ The properties of enzyme reactors in flow systems have been investigated by us for glucose,¹¹ urea¹² and L-aminoacid oxidase¹³ and for chromatographic peaks.¹⁴ This study reports on the properties of a combined system using a FIA technique, an on-line dialyzer, and on-line enzyme reactors. The technique was applied to the analysis of glucose and urea using a spectrophotometric and an electrochemical detector, respectively.

EXPERIMENTAL

Flow injection system

The flow systems for glucose and urea determinations are shown in Fig. 1. The components consisted of a Gilson 4-channel peristaltic pump (Minipuls 2), a Bifok 54 cm dialyzer, an EIL ammonia flow-through electrode, and a McPherson spectrophotometer with a flow-through cuvette. The peak values of both the

spectrophotometer and the pH-meter were read digitally in addition to the analogue recording. The smallest relative standard deviation (0.12% for 10 measurements) was obtained with an integration time of 100 ms on the spectrophotometer. The total dead volume of the cuvette was 100 μ l, 34 of which were in the 10 mm light path. The tubings were made of Teflon, i.d. 0.3 mm with Altex fittings. The sample was introduced by a rotating valve with a bypass loop¹⁵ (Fig. 1 C). For this study the sample was introduced with a syringe into a sample loop of a 0.8 mm i.d. Teflon tubing, but for real applications the fourth channel of the pump could have been used. The length of the sample loops were varied as described in RESULTS to obtain sample volumes of 25, 50, 100, 150 and 200 μ l. The sample loop gives a plug-formed sample-profile at the site of the injection. The dialyzer consisted of two halves screwed together with either an 11.5 or 17 μ m thick membrane of Cuprophane in between. Rectangular grooves, 0.1 mm deep and 1.5 mm wide, length 540 mm, were cut in each half giving a membrane area of 8.1 cm². The dialyzer and the enzyme reactor were immersed in water thermostatted at 25.0°C.

0.1 M phosphate buffer, pH 6.0, was pumped through both the upper channels in Fig. 1 A. The reagent solution contained 2.9 mM 4-amino-phenazone, 0.1 M phosphate buffer, pH 6.0, and it was saturated with N,N-dimethylaniline. When phenol was used instead of N,N-dimethylaniline, its concentration was 42 mM. The buffer bottle was kept at 50°C with oxygen bubbling for about 5 min. Otherwise microbubbles of air interfered with the spectrophotometric measurement.

50 mM Tris-HCl buffer, pH 7.0. was pumped through both buffer channels in Fig. 1 B. The base was 0.5 M NaOH.

Enzyme reactors

Glucose oxidase (EC.1.1.2.3) from *Aspergillus niger* (Serva 22741) was immobilized on porous glass, Corning GPG-10, 80-120 mesh, pore diam 729 Å, using glutaraldehyde as described before.¹¹ 60 mg enzyme were added per gram of glass, 77% bound to the glass. The reactor was a Teflontube, i.d. 3 mm, volume 200 µl. The tube was threaded inside at the ends to fit the Altex connectors. The porous enzyme-glass was kept in place with polypropylene gauze at the ends. A circular piece was cut with a corc bore, it was woven with 8 threads per mm, the openings being 0.02-0.03 mm.

Peroxidase (EC.1.11.1.7) from *Horse radish*, (Sigma P8375) was immobilized in the same way taking 60 mg enzyme per gram glass, 22% of the enzyme bound to the glass. The reactor size was either 200 or 400 µl.

Urease (EC.3.5.1.5), from *Jack beans*, (Sigma U4002), was immobilized as described before¹² using 60 mg enzyme per gram glass. 55% of the enzyme bound to the glass. The reactor volume was 300 µl.

RESULTS AND DISCUSSION

Optimization of the flow system

The length of the bypass loop of the injector was varied when 150 μ l 3 mM of an inert model substance, catechol, was taken as a sample. The set-up was as in Fig. 1 A, except that the detector was placed immediately after the dialyzer. The peaks were broadened up to 10% at a loop length of 10 cm, and 3% at 20 cm. A loop length of 60 cm was used in the following.

Fig. 2 shows the effect of the sample size on the signal. The steady state signal is also shown for comparison (2500 μ l). Almost half of the dispersion originates from the dialyzer, the remaining contributions come to about the same degree from the detector and injector. A sample volume of 150 μ l was found to give the optimal relation between sensitivity and dispersion. The carry-over was zero if space between the injections was 45 sec or more at a flow rate of 1.0 ml min⁻¹ in both channels. It was 9% at a spacing of 20 sec.

The mutual influence of the sample line and the detector line of the dialyzer was studied by varying the flow rates one at a time and keeping the other at 1.0 ml min⁻¹. If only the peak height was considered the pumping speeds should be 0.3 ml min⁻¹ for the sample line and 1.0 ml min⁻¹ for the detector line. When also the effect on the peak broadening was considered, a flow rate of 1.0 ml min⁻¹ in both channels seemed to be the most advantageous. The dialysis membrane is sensitive to large differences in pressure between the channels and equal flow rates are therefore to be preferred. The tubings between the

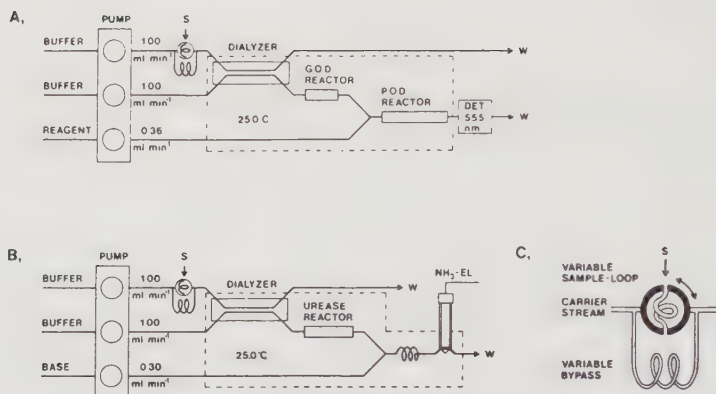


Fig. 1 FIA systems for detection of (A) glucose and (B) urea. The injector is schematically shown in C. For detailed explanation, see text.

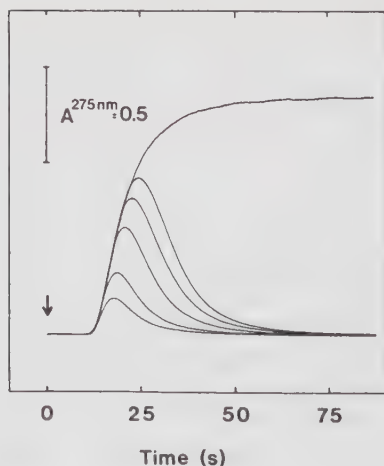


Fig. 2 Response curves obtained by injecting 25, 50, 100, 150, 200 and 2500 μl of 3 mM catechol. Set up as in Fig. 1 A, with the detector in place of the GOD-reactor.

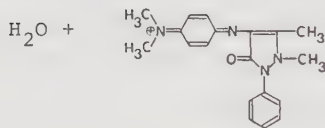
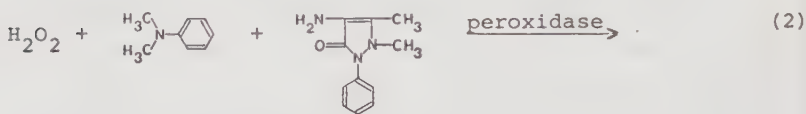
dialyzer and the detector cell plus the enzyme reactors will give some overpressure in the detector channel. It will result in a small liquid flow through the membrane pores towards the sample side. The flow counteracts clogging by macromolecules on the sample side.

Two membranes were tested, with a flow rate of 1.0 ml min^{-1} in both the sample and detector channels. 16.0% were transferred through the $11.5 \text{ }\mu\text{m}$ membrane when a solution of catechol was pumped through the sample line. The transfer through the $17 \text{ }\mu\text{m}$ membrane was 13.4% under the same conditions. The concentration dependence was tested with injections of various sizes. The calibration curves were strictly linear ($r=0.9999$) over the range covered by the spectrophotometer. This is to be expected since the dialyzed fraction should be concentration independent for each solute if the time, temperature and volume is kept constant. There will of course be an appreciable back diffusion from the detector side when the dialyzed fractions are as high as those given above.

Fig. 3 shows the dispersion of a flow system containing the dialyzer, simulated enzyme reactors of various sizes and the spectrophotometric detector. The reactors were filled with porous silanized glass without any enzyme. It can be seen that the dispersion increases with reactor size. Reactors larger than $300 \text{ }\mu\text{l}$ contribute more to the total dispersion of the system than the dialyzer. If smaller reactors are used the largest contribution comes from the dialyzer.

Determination of glucose

Most clinical glucose determinations utilize a colour reaction coupled to the hydrogen peroxide produced by glucose oxidase. The reactions used in this study were



o-Dianisidine is the most used colour reagent but it was ruled out in the present method as it adsorbed strongly to the porous glass. 4-amino-phenazone which is one of at least 30 compounds reported for glucose determinations, was tested and there was no problem from adsorption. The most used coupling reagent for this compound is phenol but N,N-diethylaniline has also been used.¹⁶ The product formed with N,N-dimethylaniline had a higher molar absorptivity, $18 \cdot 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ at 555 nm than that obtained with phenol which was $7 \cdot 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ at 505 nm. Compounds of this type are considered to be less affected by interferences from other substances in serum than o-dianisidine.

The flow dependence of the colour reaction (2) with a 200 μl peroxidase reactor in Fig. 4. Measurements were made with a steady state supply of hydrogen peroxide (0.074 mM after mixing)

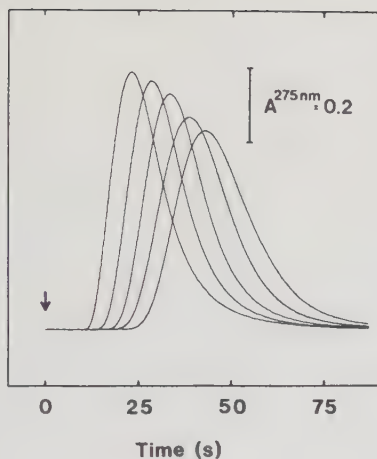


Fig. 3 The dispersion of an 150 μ l 3 mM catechol sample with 0, 100, 200, 300 and 400 μ l enzyme reactors in the flow system. Set up as in Fig. 1 A, but with the detector immediately after the GOD-reactor.

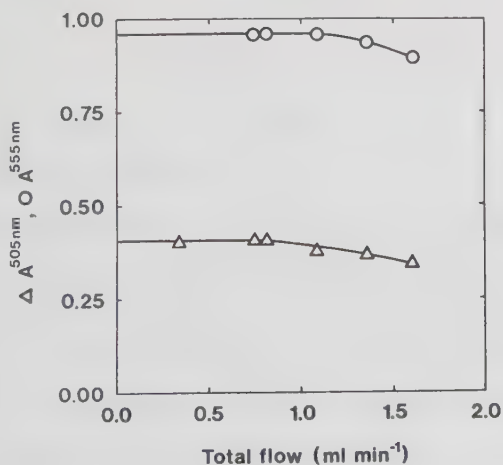


Fig. 4 Flow dependence of colour formation in reaction (2). POD reactor volume 200 μ l. Coupling reagent: (O) N,N-dimethylaniline or (Δ) phenol.

The ratio between the sample and the reagent flow rate was 1:0.36 in this experiment as well as in all following. It can be seen that the absorbance of the colour complex formed with N,N-dimethylaniline is almost three times higher than that formed with phenol. The reaction rate in the reactor is also higher with the aniline since 100% conversion efficiency can be upheld at higher flow rates. If a reactor volume of 400 μl is selected for the final analytical system there will be some reserve capacity since the total flow rate will be 1.36 ml min^{-1} .

Hydrogen peroxide samples were next injected into a FIA-system, in which the dialyzer had been removed. A linear relation was found between the concentration of H_2O_2 in a 150 μl sample and the peak absorbance in the range 10^{-6} – $3 \cdot 10^{-4} \text{ M H}_2\text{O}_2$. The slope in a log-log plot was 1.009, ($r=0.99992$) i.e. the reaction was strictly stoichiometric. The calibration curve starts to bend just after $3 \cdot 10^{-4} \text{ M H}_2\text{O}_2$. This bend is caused by a decrease in the conversion efficiency of the enzyme reactor. The conversion efficiency could have been increased by increasing either the size of the reactor or the reagent concentration as both were found to contribute to the decrease. This was not done, however, since the spectrophotometer would then impose a limit. The absorbance was 1.3 abs. units at $3 \cdot 10^{-4} \text{ M H}_2\text{O}_2$.

Glucose samples were next injected into the apparatus shown in Fig. 1 A, i.e. the system contained the dialyzer, a 200 μ l glucose oxidase reactor and a 400 μ l peroxidase reactor. The calibration was made using two sample sizes (Fig. 5). A comparison was also made with the steady state level. The method measures the concentration of β -D-glucose but a second scale for total glucose is added for convenience. It is made with the assumption that 63.5% of the glucose is β -D-glucose. The reported results were obtained at 45 samples hour⁻¹ so that the carry-over should be negligible. The glucose (total) range of clinical interest is 2-16 mM for blood, the normal fasting value is 4.2 mM. The range for urine samples is normally 0.1-1 mM; an excess indicates malfunctions. The range of the method will thus cover the concentrations of clinical interest if 25 μ l samples are taken. The sensitivity can be adjusted upward or downward by changing the sample size, the thickness of the dialysis membrane and the flow rate. Table I shows the results when two reference sera were analyzed.

Urine samples will produce a transient in the spectrophotometric cell due to the difference in ionic strength between the buffer and the sample. The method can easily be adapted for urine analysis if the ionic strength of the buffer is increased by the addition of phosphate and sodium chloride. The ionic strength of the samples should also be normalized by suitable additions.

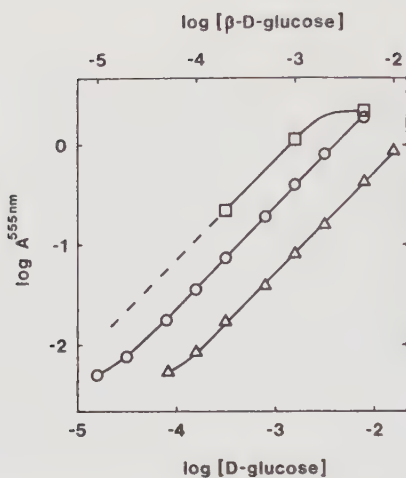


Fig. 5 Calibration curves obtained with the flow system in Fig. 1 A. Sample volumes (Δ) 25 μ l and ($\circ$) 100 μ l. Steady-state measurements are shown by ($\square$).

TABLE I

Analysis of reference sera from a calibration curve or with standard additions.

Sample	Concentration of glucose (mM)		
	Nominal	Calibration curve	Standard addition
Pathonorm B, serum	1.94 ^x	1.73	1.59
Serum Technicon	12.4 ^{xx}	11.8	12.1

^xRange 1.66-2.22 mM

^{xx}Mean of three different methods

Determination of urea

The ammonia electrode shows a concentration dependent memory effect¹⁷ which has to be taken into account in addition to the carry-over. The memory effect may prevent the determination of a sample of low concentration following immediately after a sample of high concentration. If the time is long enough to give separate peaks, the second reading will be correct. This is in contrast to the additive effect of the tail when there is a real carry-over. Fig. 6 illustrates the ammonia electrode behaviour when equal samples were injected with varying spacings.

Fig. 7 shows the determination of ammonia and urea. A comparison between a steady state system without dialyzer (black squares) and with dialyzer shows that 27% is dialyzed both for ammonia (black triangles) and urea (white triangles). The enzyme reactor does not give complete conversion above 0.05M urea. The levelling off of the upper curve depends on a pH change due to the large amount of NH_4Cl added.

A FIA analysis of ammonia (black circles) using 100 μl injections results in a response which is 35% of the dialyzed steady state level. The dynamic response for urea (white circles) is 27% of the steady state level measured after the dialysis (white triangles). There is a slight downward bend for the dynamic curves (circles) which is caused by increased response times of the electrode at low concentrations.¹⁷

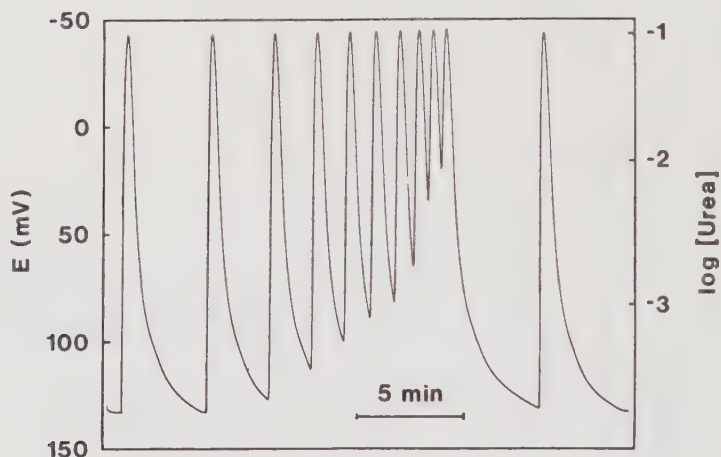


Fig. 6 The negligible influence of electrode memory effect on the result with the urea detection system (Fig. 1 B). All samples were 25 μ l 100 mM urea. The smallest spacing is 35 s.

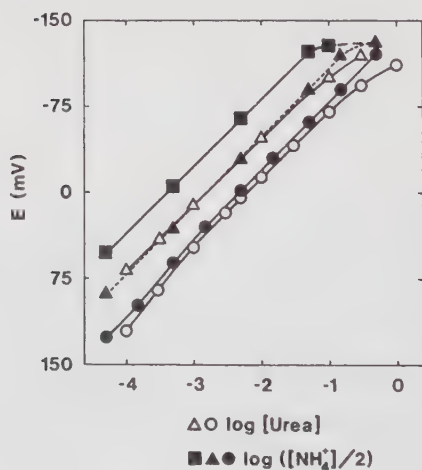


Fig. 7 Calibration curves for urea and ammonium in the urea detection system (Fig. 1 B)

O ● with FIA of 100 μ l

Δ ▲ with steady-state measurements

■ with steady-state measurements without dialyzer

The difference between the urea and ammonia lines (circles) is caused by a different dispersion of the two. Adams and Carr⁹ also observed different time courses of dynamic urea and ammonia profiles after passage through a urease reactor. The difference in peak shape in our system was much less than that reported by the mentioned authors. The mechanism of the peak broadening has not been investigated and in our system the dialyzer may also contribute.

The range of interest for clinical serum determination of urea is 3.3-9.7 mM. It can be seen from Fig. 7 that the calibration curve is strictly linear in this range (white circles).

The urea concentration of urine is normally 0.33-0.58 M. In order to optimize the method for urine determinations the sensitivity should be reduced, e.g. by taking smaller samples. There may be effects¹⁷ on the electrode if the ionic strength in the outer solution is much different from that of the internal filling solution but the effect is somewhat smaller in flow injection runs than in a continuous immersion. The effect of changes in ionic strength is much smaller for the electrode than for the spectrophotometer in the method described above. There should also be an ion strength effect on the dialyzer but its size was not determined. In order to reduce all these effects further, the sample can be diluted so that the ionic strength is normalized.

DISCUSSION OF THE COMPLETE SYSTEM

The presence of serum proteins or sediments from urine sample interferes more or less with all detection methods. In spectrophotometric methods pronounced interferences are caused by light scattering, adsorption on the windows, and reaction with the reagents to produce coloured compounds. Gas membranes for electrodes tend to be clogged by precipitates and adsorption to the walls may cause ion exchange effects. Clogging of the dialysis membrane may also occur with time but it is simpler to clean this single component than the whole analytical system. A proper design with a slightly higher pressure on the detector side of the dialyzer reduces clogging of the pores.

Hansen et al¹ stated that dialysis turned out to be the least practical approach (in flow injection analysis). The improvement reported here on the dialysis step comprises a five times larger surface (8 or 30 times larger than ref. 5) at the expense of a doubling in the dead volume. The dialyzed fraction then becomes an order of magnitude larger and the lack of sensitivity noted earlier¹ is now remedied. From a practical point of view a replacement of membrane is done very quickly. The dialyzer represents a dispersing element in the flow system but it has been shown that it can be balanced against the dispersion of other components of the system. As can be seen from Table I there were up to 9% difference between the two methods of evaluation. Ionic strength and viscosity effects are the main factors behind the difference. The procedure required to remove these sources of error depends on the intended application and the detailed design of the system.

The pros and cons of enzyme reactors versus a FIA-system using soluble enzyme can only be correctly evaluated when each of the systems has been optimized for the same application. The reactors used in this work are unnecessarily large for samples containing low substrate concentrations. In such cases the reactor sizes could have been reduced resulting in a reduced dispersion and - at least with the spectrophotometric detector - a higher flow rate and a higher sample through-put..

Complete conversion of the substrate is unpractical with a soluble enzyme. A very large amount of enzyme or a long reaction time would be necessary. A partial reaction is therefore accepted which results in lower sensitivity and a larger dependence on parameters effecting the kinetics. A soluble enzyme will interfere with the detection system in many cases.

Reactors containing immobilized enzymes can be designed so that 100% of the substrate is converted to products. The conversion then becomes independent on small or moderate changes in flow rate, temperature, pH, ionic strength, and inhibitor concentration.¹⁸ The product concentration is a strictly linear function of the substrate concentration, which is not necessarily the case with a soluble enzyme. A well-designed enzyme reactor system can produce results with a small standard deviation, for the system described above it was somewhat less than 1% for the overall determination.

The system described above can be further optimized e.g. for serum analysis. Glucose analysis can be made using a smaller dialyzer, and smaller enzyme reactors so that the dispersion decreases at the expense of sensitivity. The size of the dialyzer can also be decreased for serum analysis. In such a case another spectrophotometer has to be used and it is possible to increase the pumping speed.

The overall result will be a system which can be optimized for a much larger sample through-put. The speed of analysis of urea samples are mainly set by the electrode properties. Faster electrode constructions are feasible.¹⁹

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A POST-COLUMN ENZYME REACTOR FOR DETECTION OF OXIDIZED CHOLESTEROLS IN H.P.L.C. SEPARATIONS

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SUMMARY

An enzyme reactor is used as a post-column detector after the h.p.l.c. separation of a mixture of cholesterol and some auto-oxidation products. The enzyme reactor contains cholesterol oxidase immobilized on controlled-pore glass. A method for purification of the enzyme is described. The properties and the solvent dependence of the reactor are discussed. A post-column system for dilution of the eluent with aqueous buffer is described.

The auto-oxidation of cholesterol produces a number of hydroxy and keto derivatives. Some of the oxidation products are very active physiologically: angiotoxicity [1–3] has been proved and involvement in the arteriosclerotic process has been suspected [4]. Some of the auto-oxidation products identified are cholest-5-ene-3 β ,20 α -diol (20 α -hydroxycholesterol), cholest-5-ene-3 β ,25-diol (25-hydroxycholesterol) and cholest-5-ene-3 β ,7 α -diol (7 α -hydroxycholesterol). A major problem in connection with research in this area is the determination of minute quantities of oxidation products in the presence of a large excess of the parent compound. Furthermore, the detection of sterols and hydroxysterols after h.p.l.c. separations is difficult because of the absence of strong u.v. absorption. At the end of the usable wavelength scale, at 210 nm, a moderate u.v. absorption can be utilized and this is more advantageous than the refractive index detector [5–7]. Methods for the h.p.l.c. separation of steroids have recently been reviewed [8]. Derivatization of hydroxysteroids before separation has been reported [9]. Several papers have been concerned with the reactions of keto steroids. A highly sensitive post-column reaction giving fluorescence for ketosteroids has been described [10].

This study is concerned with the development of a post-column reactor containing immobilized cholesterol oxidase. Very many analytical applications of soluble cholesterol oxidase are known. Immobilized cholesterol oxidase has been used analytically both in enzyme electrodes [11] and in methods for the determination of cholesterol in flow systems with enzyme thermistor [12] or luminescence [13] detection. A flow-injection system based on immobilized 3 β ,17 β -hydroxysteroid dehydrogenase and u.v. detection via the NAD⁺/NADH system has also been described [14]. Enzyme reactors do not appear to have been utilized as post-column devices in h.p.l.c.

EXPERIMENTAL

Preparation of enzyme reactor

Cholesterol oxidase (2 mg) from *Nocardia erythropolis* (Boehringer Mannheim) in 1 M ammonium sulphate was used as starting material. Straightforward immobilization of this enzyme preparation gave reactors which lost activity very quickly. Even when the ammonium ions were removed by dialysis, an unstable reactor was obtained. It was suspected that membrane fragments were bound to the glass and that the enzyme diffused slowly into the solution when the detergent was added in the flow. Therefore the enzyme was first filtered through a 0.22- μ m Millipore filter; the filter was then placed in a beaker and treated with 0.5 ml of 1% (v/v) Triton X-100 solution in 50 mM Tris-HCl solution (pH 7.9) for 1 h at room temperature. This solution was filtered through a 0.22- μ m filter, and the beaker and filter were thoroughly washed with 0.15 ml of 50 mM phosphate buffer, pH 6.0. The filtrate had a pH of 7.2 and contained 60% of the original enzyme activity. The amount lost in the first filtration (6%) represents the free enzyme in the original preparation. Further treatment of the filter from the second filtration could recover another 6%. The purity of the filtrate from the second filtration was checked by electrophoresis on 13% SDS polyacrylamide-gel as described by Neville [15]; two bands were obtained in the 59000–60000 m.w. range. The original preparation gave a number of bands with especially strong color in the 69000–70000 m.w. range. The first filtrate also gave several bands. The strength of the bands at 59000–60000 was proportional to the enzyme activity in the various solutions. According to unpublished results [16], the molecular weight of cholesterol oxidase is 59000.

The enzyme in the second filtrate was immobilized on silanized porous glass with glutaraldehyde as the coupling reagent [17]. The glass was Corning CPG-10 (80–120 mesh with 729-Å pore diameter). The enzyme glass was filled into a reactor tube (i.d. 3 mm, length 45 mm). Steel reactors were used for h.p.l.c. and PVC reactors for other experiments.

Apparatus

A Spectraphysics SP 8000 liquid chromatograph with gradient facilities was used together with a Spectraphysics variable-wavelength u.v. detector. The column was prepared with C-18 ODS Hypersil (5 μ m, i.d. 3 mm, length 200 mm) and thermostated at 37°C. A 10- μ l sample loop was used. The post-column arrangement is described under Results.

A 3-channel peristaltic pump (Pharmacia Fine Chemicals, Model p3) and a Zeiss PMQ-II spectrophotometer with a flow-through cuvette were used for evaluation of the reactor properties. Samples were introduced with an Altex 4-way rotary valve (series 202). Ultraviolet spectra were obtained with a recording spectrophotometer (GC 17/McPherson Instrument, model EU-700).

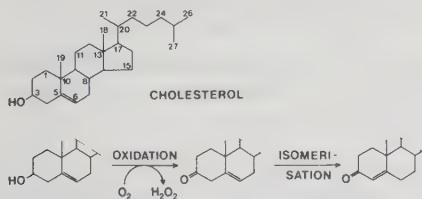
Standard substances

The sterols examined were 20- α -hydroxycholesterol and cholesterol (Sigma Chemical Co.), 25-hydroxycholesterol, 7 α -hydroxycholesterol, 7 β -hydroxycholesterol and 7-ketocholesterol (Steraloids Inc.), cholest-4-ene-3-one (Fluka Chemicals), cholestane-3 β ,5 α ,6 β -triol (Supelco Inc.) and sitosterol and stigmasterol (gifts from The Swedish National Food Administration).

RESULTS

Spectroscopic aspects

Cholesterol oxidase catalyzes the reaction



Cholesterol itself has an absorption maximum of moderate molar absorptivity at 202 nm, but it is not convenient to select lower wavelengths than 210–212 nm because of low lamp intensity and solvent absorption. The base line at 210 nm is noisy and gradient elution is usually impossible. The final product, cholest-4-ene-3-one, has a conjugated system with an absorption maximum at 241 nm and a high molar absorptivity, $\epsilon \approx 15 \times 10^3 \text{ l mol}^{-1} \text{ cm}^{-1}$.

In aqueous solutions a detergent is necessary for the solubilization of cholesterol and its oxidation products. The usual detergent, Triton X-100, is ruled out because of its u.v. absorption and therefore a 0.25% (w/v) solution of Thesit (polyoxyethylene-9-lauryl ether) was used.

Reactor properties

A 51 μM solution of cholesterol in 50 mM phosphate buffer, pH 7.2, containing 8% (v/v) ethanol and 0.25% Thesit, was pumped through the enzyme reactor. The flow rate was varied from 0.1 to 0.97 ml min^{-1} ; Fig. 1 shows the results. The conversion efficiency was calculated from the molar absorptivities determined separately. Figure 2 shows a calibration curve obtained at 1.0 ml min^{-1} when the cholesterol concentration was varied; the dotted curve represents 100% conversion. The deviation at higher concentrations follows theory for a value of the Michaelis–Menten constant of 10^{-4} M ; this value was determined for the immobilized enzyme in the reactor as described earlier [18]. The pH optimum was also determined and confirmed as 7.2. The activity at the temperature optimum, 40°C, was about three times greater than at 25°C, but decreased rapidly above 40°C. Both the

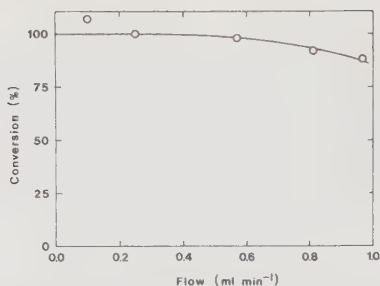


Fig. 1. Conversion of 51 μM cholesterol to cholest-4-ene-3-one in enzyme reactor. 50 mM phosphate buffer, pH 7.2 with 8% (v/v) ethanol and 0.25% Thesit.

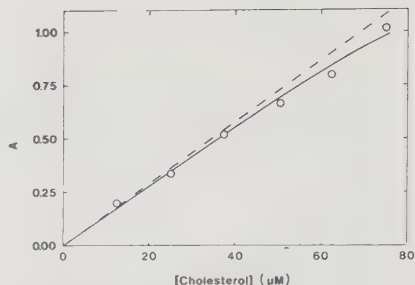


Fig. 2. Calibration curve obtained by pumping a premixed cholesterol solution (for comparison, see Fig. 1) through the reactor at 1 ml min⁻¹. Measurement at 241 nm in 1-cm cells.

type and concentration of detergent was of importance. Thesit was more advantageous than Triton X-100 or polyethyleneglycol, and the activity was very much larger when it was present. The oxygen content of the air-saturated solutions was found to be sufficient and this parameter did not affect the reaction rate up to a cholesterol concentration of 100 μM .

Figure 3 shows the activity as a function of the amount of alcohol in the solvent. It was obtained spectrophotometrically for soluble enzyme. The reactor conversion efficiency will decrease more slowly with alcohol content than that of the soluble enzyme, in accordance with the predictions of the equations relating reactor performance to the constants of enzyme kinetics [18]. The amount of enzyme, i.e. the reactor volume for a given specific activity, affects the conversion efficiency, of course.

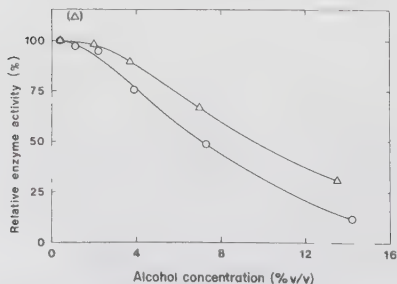


Fig. 3. Relative enzyme activity with soluble cholesterol oxidase. The cholesterol concentration was 0.35 mM in 0.5 M phosphate buffer at pH 7.5 with 0.7% Thesit, 4 mM H_2O_2 and 160 IU ml⁻¹ catalase. (○) 1-Propanol; (Δ) ethanol.

TABLE 1

Conversion of sterols with soluble enzyme and with two different flow rates through an enzyme reactor

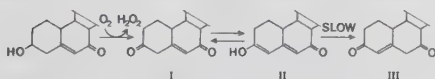
Substance (50 μ M)	Conversion in enzyme reactor (%)		Relative activity in solution (%)
	0.5 ml min ⁻¹	1.0 ml min ⁻¹	
Cholesterol	98	84	100
20 α -Hydroxycholesterol	99	94	142
25-Hydroxycholesterol	99	91	99
7 α -Hydroxycholesterol	81	61	18
7 β -Hydroxycholesterol	73	52	9
7-Ketocholesterol	—	24	3
Cholestane-3 β ,5 α ,6 β -triol	—	—	<1
Sitosterol	96	80	75
Stigmasterol ^a	86	70	35

^aDifficult to dissolve; concentration 14 μ M.

Selectivity

Table 1 shows the activity for some sterols with the enzyme reactor at two flow rates and with enzyme in solution. The enzyme has a broad activity for a number of sterols. It is important that the 3 β -hydroxy group is present and there should not be any substituent at the 4- or 5-position; other substituents in this part of the molecule affect the activity to varying degrees. Substituents in the side chain are of less importance.

The selectivity of u.v. detection of 7-ketocholesterol in a chromatographic system is more complicated than that for the other sterols. Its absorption maximum is at 236 nm and the molar absorptivity at 241 nm is about 10^4 l mol⁻¹ cm⁻¹. Separate steps can be observed in the reaction:



Compound (I) is rapidly rearranged to (II) which has an absorption peak at 322 nm ($\epsilon \approx 1.1 \times 10^4$ l mol⁻¹ cm⁻¹); the absorbance of compound (II) at 241 nm is substantially less than that of the 7-ketocholesterol itself ($\epsilon \approx 10^3$ l mol⁻¹ cm⁻¹). The rearrangement to (III) requires several days for completion at room temperature. Compound (III) has an absorption maximum at 230 nm. Absorption bands were calculated according to the rules for the absorption of α,β -unsaturated ketones and aldehydes [19]; this resulted in an absorption peak for compound (II) in the range 330–338 nm which is sufficiently close to the band observed at 322 nm to give support to the reaction. The response at 241 nm will be very dependent on the conversion

efficiency, and quantitative work should not be based on this response. However, 7-ketocholesterol can be determined either before the reactor at 236 or 241 nm or as compound(II) after the reactor at 322 nm.

Inclusion of the enzyme reactor into the chromatographic system

The chromatographic set-up is shown in Fig. 4. The outlet from the liquid chromatograph was connected to a mixing tee made as described by Frei et al. [20]. Aqueous 65 mM phosphate buffer, pH 7.2, containing 0.32% Thesit was pumped into the tee with a high-pressure pump (Waters Inc. Model M-6000A) with a pulse damper and a pressure monitor from a high-pressure pump (LDC Model 396). The mixed flow entered a stream splitter [20], the splitting ratio being controlled by two needle valves (Nupro Model SS-1SA). A slight back-pressure in the detector could be set by another needle valve. All connections were made with 0.5-mm i.d. stainless steel tubing. The mixer and stream splitter were made of steel, with 0.5-mm bore.

Ethanol-water (75 + 25) was selected as the mobile phase. The nitrogen in the buffer was first expelled with oxygen. The buffer was then heated to near 100°C to reduce the partial pressure of oxygen. The oxygen content (2.30 ml of O₂ STP/100 ml of water at 100°C) is somewhat higher than the solubility of oxygen in an air-saturated buffer at 25°C, which was shown above to be sufficient for the enzyme reaction. The mobile phase in the SP 8000 chromatograph was degassed with helium. This treatment effectively prevented bubble formation in the mixed solvent.

The enzyme activity decreases rapidly when the content of organic solvent increases (Fig. 3). Ethanol contents higher than 30% rapidly and irreversibly degrade the activity of the enzyme reactor. Even a somewhat lower ethanol concentration causes a slow denaturation. A high water content of the mobile phase, however, leads to unacceptably long retention times for the less polar sterols. Dilution with buffer is therefore necessary to bring down the ethanol content before the reactor. The optimum dilution ratio is found by balancing the loss of sensitivity caused by dilution against the decreased conversion efficiency with increased ethanol content in the reactor.

A diversion was arranged in order to study the optimum flow through the enzyme reactor at a given total flow. If an ideal reactor giving 100% conversion had been used, the response should be independent of the splitting ratio. As shown in Fig. 1, the conversion decreases with increased flow rate and a better response should therefore be obtained if the total flow through the reactor is low.

The most important aspect to be considered in the optimization was found to be the base-line stability. If the two pump systems pulsate, the mixing ratio of solvent and buffer will vary with time. If the solution is inhomogeneous radially in the tubing after the mixing tee, a diversion, especially a small one, may amplify the variations as a non-representative sample is taken. It was found that a restrictor and a pulse damper in the buffer line were essential. The SP 8000 pump was run in the constant-

pressure mode in order to reduce the flow fluctuations. It was also found that the flow through the reactor could not be less than about 40% of the total flow; otherwise, the base-line instability became objectionably large. The base-line instability was caused by variations in the refractive index in the detector cell. The amplitude was independent of the wavelength setting and the frequency of the rather regular variations was affected by the splitting ratio.

When the limitations in the splitting ratio and in the reactor flow rate are taken into account, the total flow should be as low as possible. However, low flow rates will increase the elution time. The following compromise was selected. The mobile phase flow rate was 1.5 ml min^{-1} and the buffer flow rate 5.0 ml min^{-1} . A splitting ratio of about 2:3 gave a final flow through the reactor of 2.7 ml min^{-1} and an ethanol content of 17.3%. The pressure in the mixing tee was 2.9 MPa (400 psi).

A mixture of cholesterol and three hydroxycholesterols was separated with the results shown in Fig. 5. Chromatogram A was obtained at 241 nm when the flow had passed the enzyme reactor. Chromatogram B was obtained at 211 nm under identical conditions. The absorbance originates from both the unreacted and the reacted sterols, as the latter also absorb at 211 nm, although to a smaller extent than the parent compounds. Chromatogram C was obtained at 211 nm when the enzyme reactor had been removed. The

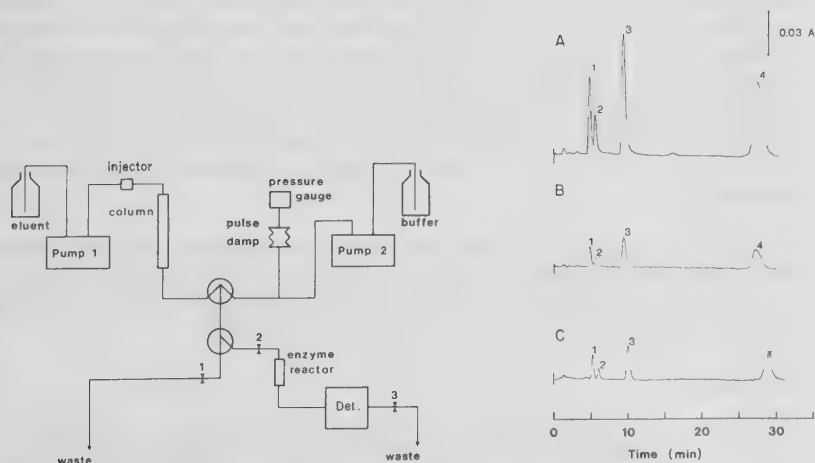


Fig. 4. Chromatographic set-up; for explanation, see text. 1, 2 and 3 are needle valves.

Fig. 5. Separation of a mixture of sterols containing (1) 25-hydroxycholesterol, $18.3 \mu\text{g}$; (2) 20α -hydroxycholesterol, $7.9 \mu\text{g}$; (3) 7α -hydroxycholesterol, $33.1 \mu\text{g}$; (4) cholesterol, $53.3 \mu\text{g}$. (A) absorbance at 241 nm after enzyme reactor; (B) absorbance at 211 nm after enzyme reactor; (C) at 211 nm without enzyme reactor.

sensitivity is improved by a factor of 3.6–4.4 in chromatogram A. The conversion factors were calculated from the known molar absorptivities, being 72% for cholesterol and 60% for 7 α -hydroxycholesterol. The peak broadening in the enzyme reactor was negligible, although a slight distortion of the cholesterol peak can be seen. It was also found, by injecting two different amounts of sample, that the peak area for each component was proportional to the concentration. The range of the u.v. monitor was 0.2 absorbance units for full-scale deflection.

Some preliminary runs with gradient elution were attempted. Monitoring at 211 nm is of course impossible under these conditions, but the base line at 241 nm with the reactor in the line was still very stable. In order to use this possibility, either the enzyme reactor should contain excess activity or the gradient should be very reproducible, as the conversion factor under the present conditions was strongly dependent on the ethanol content.

CONCLUSIONS

The improvement in sensitivity obtained with the enzyme reactor is 3.6–4.4 times if monitoring at 241 nm with the reactor is compared to monitoring at 211 nm without the reactor. If the amount of enzyme were increased so that the conversion efficiency becomes close to 100%, the sensitivity would increase further up to 6–7.5 times. An increased volume of the reactor would of course add to the column peak broadening but in the present application this might not be too important. The reactor can also be designed to cope with the total flow, thus making the splitting arrangement unnecessary. The selectivity is also increased with the enzyme reactor as there are a number of u.v.-absorbing interferences at 211 nm in a lipid sample, e.g. from foods. It is also possible to compare chromatograms at 241 nm with and without the reactor in order to obtain information about the sterol content.

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DETERMINATION OF TRACES OF MERCURY(II) BY INHIBITION OF AN ENZYME REACTOR ELECTRODE LOADED WITH IMMOBILIZED UREASE

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SUMMARY

Mercury (II) was determined in the range 0–0.7 nmol using urease immobilized in an enzyme reactor. Mercury inhibited the splitting of urea into bicarbonate and ammonia, the concentration of the latter being measured by an ammonia gas electrode. The sample volumes were 5 or 25 ml and the total mercury concentrations were 0–150 and 0–30 nmol l⁻¹, respectively. The reactor was regenerated by thioacetamide and EDTA between the samples. A derivation of the response equation showed that the inhibition should be a linear function of the amount of mercury and this was also found experimentally. The mercury was strongly bound to the urease, so the method should be useful for determination of free as well as complexed metal ions. Only silver and copper interfere.

It is well known that even trace amounts of metals and some other compounds may inhibit enzymatic reactions. A few cases have been reported in which the inhibition has been used analytically for the determination of inhibitor. A well known example is the determination of nerve gas and pesticides by the inhibition of choline esterases. Only a few reports have appeared concerning the determination of metals by enzyme inhibition. In most of these studies, urease has been used as a model enzyme.

The inhibition of urease by various metals has been studied systematically by Shaw [1] and Shaw and Raval [2]. A comparison between the inhibition of urease and the tendency of the metals to form slightly soluble sulphides has been made [3]. Torén and Burger [4] used a pH-stat kinetic method for metal determination; silver could be determined in the range $2-10 \times 10^{-8}$ M with a maximum error of 0.5 ng. A thermometric titration has been used for the determination of 1 ng Ag(I) ml⁻¹ in solution [5]. Cadmium(II) in solutions containing 1–10 µg/10 ml has been determined by potentiometric titration [6].

Inhibition of enzymes has been used for the detection of metals in thin-layer chromatography. Urease provides detection limits in the range 3–0.02 µg of metal [7]. The lowest detection limits are obtained for mercury (0.02), copper (0.06), and silver (0.13). The method can also be used for organic mercury compounds [8] with detection limits of 0.03 µg for methoxy-

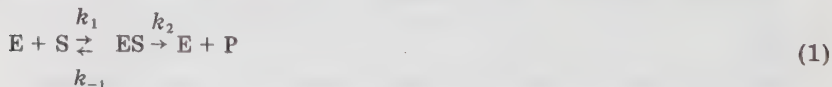
mercury chloride and phenylmercury acetate. A semi-quantitative visual agar-plate method with urease for mercury [9] provides a detection limit of 0.1–0.2 $\mu\text{g Hg(II)}$.

Inhibition of the glucose–glucose oxidase system can also be used for metal determinations but with substantially higher detection limits [10]: $2\text{--}8 \times 10^{-5}$ M for mercury(II) and $2\text{--}10 \times 10^{-6}$ M for silver(I). Carbonic anhydrase inhibition gives a sensitivity [11] of about 2×10^{-4} M for silver nitrate. Mercury(II) (5×10^{-7} M) inhibits the glucosidase system, and this can be used for electrochemical detection [12]. Removal of the metal from a metalloenzyme can also be utilized analytically. The determination of zinc in the range $5\text{--}50$ $\mu\text{g ml}^{-1}$ with aminopeptidase can be cited as an example [13].

There should be some advantages in using the recently described enzyme-reactor electrode systems [14, 15] for metal determination and the present investigation was undertaken to evaluate the possibilities. Urease was selected because the technique of immobilization is well established, the immobilized enzyme is stable over long periods of time, the reaction products can be measured with very high selectivity, and the enzyme is very sensitive for traces of metals.

THEORY

An enzyme, E, is supposed to combine with a substrate, S, to a substrate–enzyme complex ES. This complex decomposes to a product P, the back-reaction of which is assumed to be negligible.



The kinetics of this type of reaction has been derived by Michaelis and Menten [16]

$$\frac{dp}{dt} = k_2 e_0 (s_0 - p) / (K_m + s_0 - p) \quad (2)$$

$$K_m = (e_0 - es)s/es = (k_{-1} + k_2)/k_1$$

(Lower case letters stand for concentrations, e_0 denotes the total concentration of enzyme, and s_0 the initial substrate concentration, t is the time and dp/dt the rate of product formation.) This theory has been applied to enzyme reactors, e.g. by Goldstein and Katchalski [17]. Their derivation can be extended to cover the present application. Suppose that a substrate with initial concentration s_0 is pumped through a reactor with an area A and length l , at a flow rate of q ml min^{-1} . Equation (2) will be valid for any position in the reactor if the time variable is replaced by the length co-ordinate l_x .

$$q \cdot t = l_x \cdot A \quad (3)$$

Equation (3) is substituted into eqn. (2) which is integrated over the length of the reactor

$$e_0 k_2 \cdot A \cdot l / q = p + K_m \ln [s_0 / (s_0 - p)] \quad (4)$$

The quantity $e_0 A l$ gives the amount of active enzyme in the reactor. If an inhibitor is introduced and bound irreversibly to some of the enzyme, only the quantity $(e_0 - e_i) A l$ of active enzyme remains. The amount of inhibited enzyme, $e_i A l$, should be proportional to the amount of inhibitor.

If the product concentrations before the inhibition, p_e , and after inhibition, p_i , are measured, the amount of inhibited enzyme can be found

$$[e_0 A \cdot l - (e_0 - e_i) A \cdot l] k_2 / q = p_e - p_i + K_m \ln [(s_0 - p_i) / (s_0 - p_e)] \quad (5)$$

It is easier to study the behaviour of this equation if the conversion efficiency, $\eta = p/s_0$ is substituted into eqn. (5).

$$e_i \cdot A \cdot l = \frac{s_0 q}{k_2} \left[\eta_e - \eta_i + \frac{K_m}{s_0} \ln \left(\frac{1 - \eta_i}{1 - \eta_e} \right) \right] \quad (6)$$

The left-hand side of eqn. (6) represents the amount of metal for the present system. The square brackets on the right-hand side contain three terms; the first, η_e , is measured before a metal sample is introduced into the reactor and the second after the sample has passed through the reactor. The third term is logarithmic in these two measurable quantities. The influence of this term is determined by the Michaelis constant for the enzyme and the initial urea concentration. The amount of metal can thus be evaluated from a measurement of η_e and η_i if the constants are known. They can be collectively determined by calibration with metal solutions of known strengths.

In order to study the type of response curves to be expected, some simplification is desirable. For small values of η , eqn. (6) can be expanded

$$e_i \cdot A \cdot l = \frac{s_0 q}{k_2} (\eta_e - \eta_i) \left[1 + \frac{K_m}{s_0} + \frac{K_m}{s_0} \frac{(\eta_e + \eta_i)}{2} + \dots + \right] \quad (7)$$

The amount of inhibited enzyme is in linear relation to the quantity $(\eta_e - \eta_i)$ for large values of s_0 , i.e. when $s_0 \gg p$ and $s_0 > K_m$.

A more detailed study of eqn. (6) was undertaken by means of computer simulation. The percentage inhibition of the enzyme is given by $(\eta_e - \eta_i)100/\eta_e$. This quantity was plotted versus $e_i A l k_2 / q$, which should be proportional to the amount of metal. The results obtained in the computer plots are shown in Fig. 1. Curve *a* represents the maximum sensitivity which can be obtained in a theoretical enzyme system; curves *b*–*e* are based on the K_m value of urease as determined earlier in an enzyme reactor [18], and on the use of reasonable concentrations of urea. The sensitivity, which can be defined as the slope of the plot in Fig. 1, decreases when the ratio K_m/s_0 increases. For a given K_m , a high substrate concentration is advantageous. There is an increased curvature in the response curves obtained at high values of η_e . A number of computer plots (not shown) indicated that the linearity was

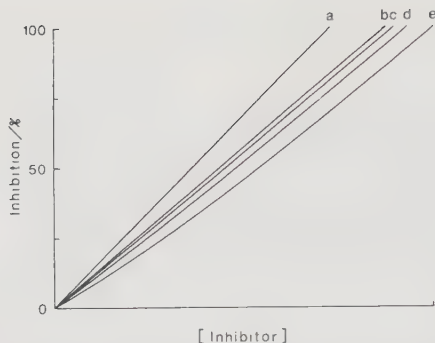


Fig. 1. Computer plot of eqn. (6) showing the percentage inhibition as a function of the inhibitor concentration in a sample of fixed size. Curves (a)–(e) are based on the constants; (a) $K_m/s_0 = 0.007$ $\eta_e = 0.01$, (b)–(e) $K_m/s_0 = 0.2$, $\eta_e = 0.01, 0.25, 0.50, 0.75$.

fairly good up to $\eta_e = 0.25$ even when $s_0 = K_m$. For $s_0 = 20K_m$, the linearity was good up to $\eta_e = 0.75$.

EXPERIMENTAL

Flow system

A schematic diagram is shown in Fig. 2. A two-channel pump (Pharmacia Fine Chemicals, Model P3) was fitted with 2.1-mm tubing and the motor speed was set so that the flow in each channel was 18.7 ml h^{-1} . A buffered urea solution was first pumped through an ion-exchange column (Chelex-100, 100-200 mesh, Na-form, i.d. 10 mm, length 95 mm) to remove traces of metal ions from the solution. The solution was then passed through the sample loop (Altex 4-way Rotary Valve, series 202) and next either through the enzyme reactor to a mixing T-joint or through a bypass directly to the T-joint. At the T-junction, the solution was made alkaline with a solution of 0.1 M NaOH containing 1 mM EDTA. The neutralization heat was removed in a heat-exchanger coil (Teflon tubing, 650 mm long, i.d. 0.5 mm, volume $140 \mu\text{l}$). The ammonia concentration was determined with a flow-through ammonia electrode (EIL, Model 8002). The potential was measured with a pH meter (Radiometer, Model PH 640) and a potentiometric recorder in parallel.

Enzyme reactor

The enzyme reactor had an i.d. of 2.5 mm and a length of 2.2 mm between the two silver frits (1/8-in. diam., 0.015-mm holes; Reeve Angel, Cat. No. LA 200). The volume of the reactor was thus $14 \mu\text{l}$. It was fitted with urease immobilized on CPG-10 controlled pore glass (Corning Glass Works, 120-200 mesh) by means of glutaraldehyde coupling as described earlier [14]. Two batches of enzyme were used, either U-1500 (4100 Sigma units/g) or

U-0251 (70 000 Sigma units/g) both made by Sigma Chemicals. Since the latter is much more active, only 3.1 mg of enzyme was taken per gram of glass, in contrast to 100 mg g⁻¹ of glass for U-1500. The procedure has been described in detail [14].

Chemicals

The sodium hydroxide solution was made from a stock solution which had been boiled to expel ammonia and decrease the blank value. EDTA was added to prevent the formation of metal—ammine complexes at the electrode.

The substrate—buffer solution was made daily by dilution of stock solutions. It was 15 mM in urea, 25 mM in maleic acid buffer, and of pH 6.15. The stock solution of maleic acid was 0.1 M, adjusted to pH 6.00 with NaOH.

The metal stock solution was 10 mM in metal nitrate and 0.1 M in nitric acid. A secondary stock solution was made daily by dilution 1000 times. Sample solutions were made by diluting the secondary stock solution with 1 mM malic acid. The malic acid solution had previously been purified from metals by passage through a column of Chelex-100. The water used was purified with a Millipore-Q system, and all glass-ware was acid-washed and then soaked in purified water. Extensive purification is necessary only in preparation of the sample solutions, as other solutions are passed through the Chelex column in the flow system when necessary. The malic acid solution was of pH 5.3; at this pH it forms weak complexes with the metal ions, so that most of the metals are present in complexed form. The complexation and the buffering action together prevent precipitation as hydroxides and reduce ion-exchange adsorption on the walls of the containers and in the flow system.

The regeneration solution used was 10 mM in thioacetamide, 10 mM in EDTA and 0.1 M in Tris, adjusted to pH 7.0 with HCl.

Procedure

A buffered substrate solution is pumped through the enzyme reactor with the rotary valve in position A (Fig. 2). The substrate concentration is selected so that only about 3% of the urea is broken down to ammonia. The ammonia concentration, which under these conditions is proportional to the enzyme activity in the reactor, is sensed by the electrode and displayed on the pH-meter and recorder. For a constant flow rate and a constant temperature, a steady-state is obtained.

Next, the valve is changed to position B and 2.5 ml of water is passed through the reactor to displace the buffer—substrate solution. Then a sample or a standard solution containing metal ions can be passed through the reactor. The metal ions may bind the enzyme and deactivate part of it. A new portion of water is passed through the reactor before the valve position is changed.

If the sample contains metal ions which deactivate some of the enzyme the conversion of urea will be lower and a lower ammonia concentration will be displayed. The difference is a measure of the metal content of the sample

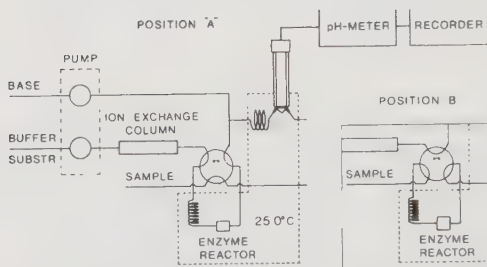


Fig. 2. Experimental arrangement for metal determinations with the enzyme reactor electrode.

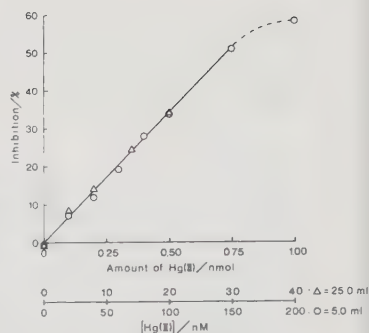


Fig. 3. Percentage inhibition of the enzyme reactor as a function of the mercury concentration for two sample sizes. A scale giving the amount of mercury in the samples is shown for convenience.

The enzyme reactor can be regenerated by turning the valve to position B and passing 3 ml of regenerating solution through the reactor followed by 5 ml of water. The system is then ready for another sample.

RESULTS AND DISCUSSION

Mercury determination

A urea substrate solution was pumped through an enzyme reactor containing 3.1 mg of urease (U-0251) per gram of glutaraldehyde glass. The valves were in position A (see Fig. 2). The output voltage from the ammonia sensor reached a steady value within 3–4 minutes. The valve was switched to position B and 25 ml of 4×10^{-9} M mercury(II) solution was passed through the reactor at an approximate flow rate of 8 ml min^{-1} . The mercury(II) in the sample is bound to enzyme molecules which then become inactive. The valve was switched back to position A and the amount of ammonia produced from the urea substrate solution was again measured with the ammonia probe. Because of the mercury(II), the ammonia concentration was then lower. The relative decrease was calculated and plotted versus the mercury concentrations when a series of 25-ml samples were run as described. The results are shown in Fig. 3.

Another series of experiments was made with sample volumes of 5 ml but with 5 times higher mercury(II) concentrations. Figure 3 is provided with a scale for the total amount of mercury in each sample, and two concentration scales for 25- and 5-ml samples, respectively. It can be seen that the relative inhibition is a linear function of the mercury(II) concentration for a given sample size, and that, if the sample size is varied, it is a linear function of the

amount of mercury. This shows that the mercury(II) in the sample is quantitatively removed from the sample and bound to the enzyme in the reactor. For more than 50% relative inhibition, the linear relationship breaks down, probably because the flow is unevenly distributed in the minute reactor bed. Enzyme beds along the main flow paths become saturated with a resultant breakthrough of mercury.

The Michaelis constant for soluble urease has been reported [19] to be 19 mM. For urease immobilized in a reactor similar to that used here, a value of 3.1 mM has been reported [18] at a flow rate of 0.44 ml min^{-1} . The substrate concentration was 15 mM; K_m/s_0 then becomes 0.2, and η_e is 0.033. Equation (6) or (7) then predicts a linear response, in agreement with the results shown in Fig. 3 for relative inhibitions below 50%. Equation (6) also predicts that the values of K_m/s_0 and η_e give about 85% of the maximum sensitivity which can be obtained with the amount of enzyme used (Fig. 1). The sensitivity is the slope of the line in Fig. 3. The only practical way of increasing the sensitivity further is, therefore, either to decrease the reactor volume or to decrease the density of enzyme on the glass spheres.

A few initial experiments were made with a different procedure. The flow of sample solution was mixed with a flow of buffer—substrate solution and pumped through the reactor. Valves were then not needed, and the inhibition could be recorded continuously. The disadvantage of this method was that the metal ions formed complexes with the ammonia produced or with impurities in the substrate—buffer solution. The method was also slower and less reproducible than that described above. This procedure was therefore not examined further.

Mercury(II)—enzyme complex formation

Once mercury(II) had been bound to the enzyme, large amounts of substrate—buffer solution could be passed through the reactor without changes in the relative inhibition. It can therefore be concluded that the mercury(II)—enzyme complex is much stronger than the complexes between mercury(II) and maleic acid or ammonia.

Strong complex formers are required for regeneration of the enzyme. Various solutions (2 ml) were passed quickly through a partly (85%) inhibited reactor. The results, shown in Table 1, were normalized to that of the thioacetamide—EDTA solution, and the values reflect both kinetic and equilibrium effects. The conditional stability constants at pH 7 for the predominant mercury(II) complex were also calculated from literature data [20–23]. The enzyme concentration in the reactor is not a well defined quantity; the concentration including the volume of the glass is less than 10^{-4} M . The stability of the mercury(II)—enzyme complex must be much larger than, for example, that of the mercury(II)—EDTA complex, as a large volume of the latter can be passed through the reactor with only a small decrease in the inhibition.

At first sight, cysteine is a very efficient regeneration agent, reactivating

TABLE 1

Regeneration of the reactor by passing 2 ml of complex former in 0.10 M Tris-HCl, pH 7.0, quickly through an 85% inhibited reactor

Complex former	Conc. (mM)	Relative regeneration (%)	$\log K^a$
Thioacetamide	10	100	—
EDTA	10		
EDTA	5	5	15.5 (HgL) [20, 21, 23]
Thioacetamide	5	12	18.7 (HgL ₃) [22, 23]
Thiourea	5	3	20.9 (HgL ₃) [20, 21, 23]
Cysteine	5	620	6.4 (HgL) [21, 23]
Histidine	5	0	13.0 (HgL ₂) [21, 23]
Methionine	5	0	4.0 (HgL ₂) [21, 23]

^a K = conditional complex constant corrected for pH effects on both the ligand and the metal. The constant relates to the dominant complex shown in parentheses. The figures in brackets are reference numbers.

more enzyme than the thioacetamide-EDTA solution. The level of activity obtained with cysteine increased with the number of regenerations but the sensitivity towards mercury(II) as an inhibitor decreased rapidly. The reagent was therefore unsuitable for use with the present analytical method, as the increase in activity seemed to result from a chemical reaction rather than from complex formation with mercury(II). In contrast to the effects observed with cysteine, a regeneration solution consisting of thioacetamide-EDTA always gave a definite and reproducible level of enzyme activity. The sensitivity for mercury(II) was constant even after a large number of inhibition-regeneration cycles. EDTA is not necessary for removal of mercury(II), but it is necessary if inhibition has been caused by metals such as Ag(I) and Cu(II).

Metals other than mercury

When samples containing 0.25 and 0.5 nmol of silver(I) were run through the reactor, the initial inhibition was high, but decreased with time because the silver(I)-amine complexes were strong enough to remove the metal from the enzyme. When the recorded value was extrapolated back to the time of sample injection, the sensitivity obtained was almost the same as for mercury(II).

All the results reported above relate to urease from batch number U-0251. The following study for other metals was made with batch U-1500. No inhibition was observed with 5-ml samples which were 0.1 or 1 μ M in Ni(II), Zn(II), Cd(II) or Pd(II), when they were introduced at a flow rate of about 1 ml min⁻¹. For copper(II) the amount of inhibition depended on the flow rate: 5 ml of 1 μ M copper(II) solution produced 5% inhibition at 15 ml min⁻¹ and 25% inhibition at 0.3 ml min⁻¹. At 0.3 ml min⁻¹, a 4 μ M solution of

zinc(II) also produced some inhibition (6%). Mercury(II), however, showed no flow dependence between 1 ml min^{-1} and 20 ml min^{-1} . At 0.3 ml min^{-1} , there was a slight decrease, probably caused by adsorption on the container walls.

Under the conditions described in the Experimental, the method is selective for mercury, with a selectivity factor of 1000 and probably much more over the metals Ni(II), Cd(II), Pb(II) and Zn(II). The selectivity over copper depends on the rate of sample injection, and the factor may vary between 50 and 1000. There is initially practically no selectivity for mercury over silver, but the presence of silver can easily be seen on the recorder. The inhibition for silver(I) (and copper(II)) decreases rapidly with time.

Number of metal ions per enzyme molecule

The urea batch U-1500 was found to contain 10.0% N (Kjeldahl method). When the immobilization procedure was used with 100 mg of enzyme taken for each gram of glass, 2.6 mg N was bound to each gram of glass when a correction (0.6 mg N/g glass) had been applied for the nitrogen from the propylamine silanization. If all nitrogen-containing compounds in the enzyme batch immobilize with the same probability, 26 mg of protein should be bound to each gram of dry glass.

A reactor contains 4.7 mg of dry enzyme-glass, and if all the protein is assumed to be urease (m.w. 480 000 [24]), there should be 0.25 nmol of enzyme present. The calibration curves were similar to those of Fig. 3 when enzyme batches were prepared from U-1500. The curves were extrapolated to 100% inhibition and the amount of mercury(II) was read. Three reactors, each made from separate immobilizations, required 0.8, 0.9 and 1.8 nmol of mercury(II), respectively, for complete inhibition. Nitrogen was determined on the last reactor. The number of mercury(II) ions per enzyme molecule was then calculated to be 3.2, 3.6, and 7.2, respectively.

It has been reported that urease contains six sub-units [25]; there seem to be 3–4 sulfhydryl active sites per molecule [26]. The total number of –SH groups which can be titrated with silver nitrate was found [27] to be 109 ± 7 . It is also possible to differentiate the –SH groups according to reactivity [28]; there are 26–28 highly reactive groups, 7–9 less reactive groups and, on unfolding of the chain, another 45–50 groups. Inhibitor studies with hydroxamic acid have shown that two inhibitor molecules are bound to each urease molecule [29].

The precision of the present determination of the number of mercury ions required to inhibit one urease molecule, is low. The amount of protein was determined only for the material in the last reactor, it may be different in the others. The volume, $14 \mu\text{l}$, in the reactor cannot be precisely determined with the silver frits used. Some of the urease may be destroyed during the immobilization procedure and there may also be other nitrogen-containing material bound to the glass.

The conclusion which can be drawn is that the number of mercury ions is too low to correspond to binding even only of the more reactive $-SH$ groups. It is more likely that a small number, perhaps one or two, per sub-unit or active site is sufficient to cause inhibition of the enzyme activity.

Differences in the enzyme batches

When the activity of a reactor containing enzyme from batch U-0251 was measured before any inhibition was attempted, the value obtained was low. After inhibition with 5 ml of $10\ \mu M$ $Hg(II)$ solution and regeneration, the activity was much higher. The initial activity was only 2% of the activity obtained after the inhibition-regeneration cycle. Regeneration solution alone did not increase the activity. No corresponding phenomena were observed with enzyme from batch U-1500. The activities of the soluble enzymes were checked and found to be in agreement with the specifications given by Sigma Chemicals. A possible explanation is that an inhibitor bound to the enzyme was displaced by mercury and washed away. The differences between the batches may also have been caused by differences in the immobilization procedure; much less of the batch U-0251 enzyme was used and there may have been differences in the binding. Addition of mercury may have caused conformational changes which restored some of the activity.

Reactors made from enzyme batch U-1500 showed a slightly sigmoidal calibration curve for mercury. A small amount of metal ions seemed to be bound before inhibition could start. This batch was less pure and may have contained some impurity which formed a complex with mercury(II) stronger than the mercury(II)-urease complex. The reproducibility of the reactors made from U-1500 was less satisfactory and a longer time was required to obtain stable potential values. The inhibition was different if the same amount of mercury(II) was introduced in different sample volumes. The impurities in this batch seem to give the reactors less desirable properties.

Increasing the sensitivity

As mentioned above, the values of η_e and K_m/s_0 were selected so that the sensitivity was close to the optimum (85%). It can be seen from eqn. (6) or (7) that the sensitivity can be changed by varying the quantity $e_i A l$. In this study, reactors with $A \times l = 14\ \mu l$ were used, and it is not practical to reduce the volume further. The remaining parameter is e_i , the amount of active enzyme. As shown above, impure enzyme preparations which were less active produced various side effects so that it seems better to use less of a pure enzyme. If a small amount of enzyme is present per gram of glass, there will be more bonds between each enzyme molecule and the alkylamino groups on the glass. The activity of immobilized U-0251 urease was less than that expected from the specification of the soluble enzyme. The low concentration of enzyme used with this batch caused denaturation, some of which could be restored by a mercury inhibition-regeneration cycle. In retrospect, it might have been better to use more than 3.1 mg of

urease U-0251 per gram of glass in the immobilization procedure and to reduce e_i by other means.

One way to reduce the amount of enzyme immobilized in a given volume is to use glass with a large pore diameter. A glass with 2000-Å pore size gave 1.4 times less activity and 1.8 times higher sensitivity than the glass with 700-Å pore size used earlier; the surface area is lower per gram of glass, the larger the pore diameter. Other possibilities might be to decrease the pore size so that less enzyme can penetrate into the glass, or to dilute the enzyme-glass with a glass with an inactive surface, but these possibilities were not examined. A batch of immobilized enzyme was treated with 0.1% ethanolamine for 75 min at pH 6 and 4°C; the activity was decreased 2.3 times and the sensitivity for mercury was increased 2.8 times.

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DETERMINATION AND ON-SITE SAMPLING OF INORGANIC AND ORGANIC MERCURY IN AQUEOUS SAMPLES WITH ENZYME REACTORS

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SUMMARY

Micro reactors containing immobilized urease are used for the sampling and determination of mercury ions. When an aqueous sample is passed through the reactor, the mercury ions are collected and bound to the enzyme. The enzyme is partially inhibited and the amount of mercury ions is determined as a decrease in the enzyme activity. The enzyme activity is determined with an ammonia electrode when a urea solution is pumped through the reactor. The selectivity is very high and the operating range is 0–15 nM Hg^{2+} for 5-ml samples. The mercury can be stored for several days in the reactor before the measurements are made. Organo-mercury compounds give essentially the same response as inorganic mercury.

Many methods are available for the determination of traces of mercury. The main method, cold-vapour atomic absorption, has been the subject of a great number of papers during the last few years [1].

The enzyme inhibition method for mercury [2, 3] has some desirable properties which make further studies worth while. The instrumentation is simple, the sensitivity is very high, and as shown in this paper the method can be modified so that at least part of the sample storage problems are solved. Errors from losses or contamination during sample handling are very important for mercury and especially for organo-mercury compounds [4, 5]. The main disadvantage is that the enzymatic technique is not well established in analytical laboratories and that further work is necessary before routine application is feasible.

Principle

Immobilized urease is filled into small reactors (see Fig. 1) provided with luer end fittings. If a buffered urea solution is pumped through the reactor a fraction of the substrate will be converted to ammonia. The measurement is done with an ammonia electrode after addition of a stream of sodium hydroxide to make the solution alkaline. If the procedure is repeated with partially inhibited enzyme a smaller fraction of urea will be converted to ammonia. The decrease represents the inhibition caused by the mercury.

The enzyme reactors are easily removed from the measuring unit and can be transported to the sampling site. A sample, say 5 ml, is taken with a disposable syringe, mixed with buffer—NaCl and pressed through an enzyme reactor. Any mercury in the sample will bind very strongly to the enzyme and therefore losses will be small. The danger of contamination is also smaller than for a bottle of aqueous sample.

EXPERIMENTAL

Reactor design

The immobilized enzyme—glass (20 μ l) is enclosed in a teflon cylinder as shown in Fig. 1. The packing is kept in place with polypropylene filters, woven with 8 threads per mm, the openings being 0.02–0.03 mm. The teflon body consists of three parts which can be pressed together inside a stainless steel tube (8 mm i.d., 10 mm o.d.) using a special tool made for the purpose. The ends are made as male and female luer fittings for connection to normal disposable syringes. End caps are used during storage to prevent drying.

Various other materials were tested and found to be unsuitable for different reasons. The silver filters used earlier [2] tended to clog with time, and nickel filters corroded and produced small amounts of nickel which inhibited the enzyme if given a sufficiently long time for reaction.

Various alternative body materials caused a special type of inhibition. It could be reversed after prolonged pumping of water or buffer. The materials tested were acid-etched Delrin (acetal polymer), PVC and plexiglas, as well as various glues and solvents for the glues. Most of the difficulties were traced to peroxides from the solvents or the materials. The all-teflon construction with polypropylene filters removed all those problems.

Immobilized enzyme

Urease (Sigma U-0251) was immobilized on porous glass (Corning 729 Å, CPG-10) by means of glutaraldehyde coupling as described earlier [6]. The glass was sieved after silanization and the fractions 120–140 or 140–170 mesh were used. The urease was inhomogeneous so that if small amounts were weighed and used the reactors might have widely different activities. Therefore a large amount of enzyme was dissolved in a buffer and a suitable fraction was used. Even so, it was found expedient to make two immobilizations at the same time, one with 1 mg of urease per g of alkylamino-glass

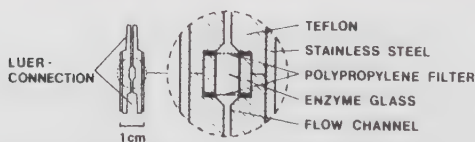


Fig. 1. Enzyme reactor. The central part is shown magnified four times.

and the other with 3 mg g⁻¹, and to use the preparation with the most suitable activity. The reactors with 3 mg of enzyme per g of glass are more stable as shown later, and should be used whenever possible. If the activity of the enzyme batch is very high, very curved calibration plots will be obtained, and to rectify this the enzyme-glass with the lower activity can be used instead. Before assessment of the final activity, 1 g of enzyme glass should be treated with 600 ml of 2 μ M Hg²⁺ solution in 1 mM malic acid (hydroxysuccinic acid) buffer pH 5.3, washed with 400 ml of regeneration solution and finally with 400 ml of water.

Sudden losses of activity either of the prepared batch or of individual reactors after the final assembly were sometimes observed. Storage in 1 mM phosphate buffer pH 7, or in buffer containing 0.02% sodium azide did not help. The most likely cause is bacterial growth and therefore care was taken to use sterile solutions and to sterilize the equipment used in the preparation. This resulted in a marked improvement. It should be worthwhile to insert filters on both sides of the enzyme bed for sterile filtration of the solution, but this possibility has not been tested.

Chemicals

Regeneration of mercury-inhibited urease was done with a solution containing 10 mM thioacetamide and 10 mM EDTA in 0.1 M Tris-HCl buffer, pH 7.0. Regeneration with sodium iodide was tested in addition to the solutions studied earlier [2], but it was found that this could only remove part of the mercury bound to the enzyme.

The substrate solution was 15 mM urea (Aristar, BDH Chemicals) in 25 mM maleic acid (*cis*-butenedioic acid) adjusted to pH 6.15 with sodium hydroxide. Traces of heavy metals in the urea-buffer solution were removed by a column (10 mm i.d., length 95 mm) containing Chelex-100 ion-exchange resin (100–200 mesh) in the sodium form (Bio-Rad Laboratories).

A stock solution was prepared from weighed amounts of mercury(II) nitrate. A secondary stock solution was made each day by diluting the primary solution 400 times. The secondary stock solution was 25 μ M in Hg²⁺. Working standards in the range 1–50 nM Hg²⁺ were prepared by taking the appropriate amount from the secondary stock solution, adding 1% (v/v) of an 0.1 M malic acid buffer, pH 5.3, and dilute with water purified in a Millipore-Q-system. The concentrated buffer had been purified by passing it slowly through a column of Chelex-100. The sodium chloride was Merck Suprapur.

Apparatus

The flow diagram and the arrangement are shown in Fig. 2. The components within the broken lines were thermostatted. The assembly was mounted on the stainless steel lid of a circulation thermostat (Julabo, Model U2). Two modes of operation could be selected with a 6-port rotary valve (Altex Model 202). In the first, called "measure", the urea-buffer solution

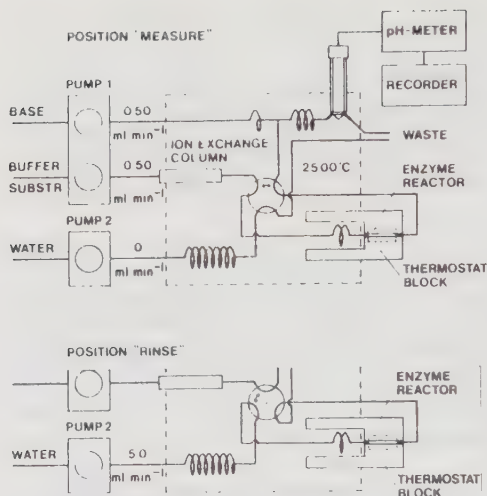


Fig. 2. Flow diagram of the measuring apparatus.

was pumped through the enzyme reactor and after mixing with 0.1 M sodium hydroxide the solution passed an ammonia electrode (EIL, Model 8002). The second position, "rinse", was used only for removing the reagents so that the reactor would be ready for use after disconnection.

The reactor is fastened on a male luer connector in the lid of the thermostat; the other connector is made from above. Heavy metal clamps can be closed around the reactor so that good thermal contact is established. The lower parts of the clamps dip into the water so that a thermal sink is obtained. If a reactor at 5°C is mounted in the clamp, its center will reach 24.5°C in 105 s and 24.99°C in 205 s if the water in the thermostat is at 25.00°C , according to theoretical calculations. In practice, temperature equilibrium is reached more quickly as thermostatted solutions are pumped through the reactor. The length of the heat-exchanger coils were adjusted until the solution reached thermal equilibrium within 0.01°C at normal pumping speed. Every turn of the coil in Fig. 2 corresponds to 200 mm. The tubings were made from teflon (0.5 mm i.d.).

In practice thermal equilibrium was reached faster than the time required for a steady-state electrode response, i.e. 4 min. The buffer-substrate and sodium hydroxide solutions were pumped with a multi-channel peristaltic pump (Ismatec, Model mp-ge).

Five successive activity measurements on the same reactor could be made with a standard deviation of 0.3%.

RESULTS

Sensitivity

The sensitivity increases if the amount of enzyme in the reactor is small. The linearity of the calibration curves will be improved when the fractional conversion of substrate, i.e. the amount of enzyme, decreases [2]. The reactors used in this study were filled with 20 μ l of enzyme glass from a lot containing either 1 or 3 mg of urease per g of glass. These amounts should give almost linear calibration curves. The sensitivities of the two batches are of the order 0.6 or 0.3% inhibition per picomole of mercury in 10 mM NaCl (3.0 or 1.5% inhibition per nM for 5-ml samples).

The sensitivity also depends on the chloride concentration of the sample (Fig. 3). The sensitivity increases up to a plateau before a final slow increase. The latter is caused by mercury in the sodium chloride solutions. At 10 mM chloride concentration, the mercury is distributed [7] between HgCl_2 (90%), HgCl_3^- (9%), HgCl_4^{2-} (1%), HgCl^+ (0.003%) and Hg^{2+} ($5 \cdot 10^{-8}\%$ or $5 \cdot 10^{-18}$ M) at a total sample concentration of 10 nM. The favoured explanation for the chloride dependence is that the presence of strong complexes decreases the activity of Hg^{2+} to such a low level that binding to weaker non-inhibiting sites is prevented. The binding to walls and to the porous glass will also decrease. It was shown, by using sodium sulphate either alone or in combination with chloride, that the effect is not caused by changes in the ionic strength. The addition of chloride increases the selectivity slightly over copper and silver.

Figure 4 shows calibration curves made with samples containing 10 mM NaCl. Besides the increased sensitivity in the presence of chloride, there is also less leakage to a second reactor placed in series with the first. There is a

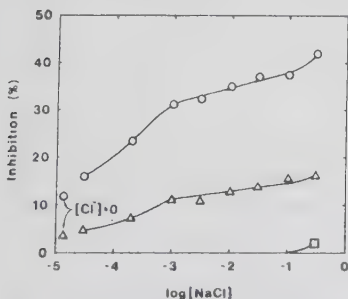


Fig. 3. Variation of the sensitivity as a function of the sodium chloride content of the sample solution. All solutions were 1 mM in malic acid buffer. (○) Samples containing 10 nM Hg^{2+} ; (△) samples containing 5 nM Hg^{2+} ; (□) blank solutions.

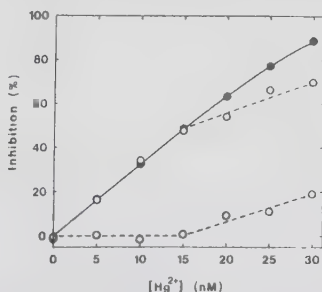


Fig. 4. Calibration curves of two enzyme reactors in series. (○) Samples containing 10 mM Cl^- : the upper curve marks the first reactor and the lower curve the second reactor. (●) The sum of the two reactors.

break-through around 50% inhibition caused by inhomogeneities in the flow. At the rapid flow rates used during sampling, there will be channeling in the packing. The presence of chloride also increases the precision of the measurements. The calibration curve, considering the sum from both reactors, is almost but not completely linear.

If two reactors are placed in series during sampling, mercury which passes the first will be collected in the second. The reactors are measured individually and the percent inhibition is calculated for each one. A correct result will be obtained if the inhibitions are added. A calibration curve made from the sum of two or more reactors can be constructed, provided that the deviations from linearity are small.

Figure 5 shows the decrease in activity with time for two reactors with different amount of enzyme. The reactors were thoroughly rinsed before the start. Nevertheless, at least part of the initial decrease is thought to be caused by desorption of enzyme not covalently bound to the glass. Part of the early inactivation and most of that occurring after one day is caused by an irreversible inactivation of enzyme molecules remaining immobilized in the bed. The effect is more pronounced for the reactor with the smaller amount of enzyme. After a week the activity stabilizes, and further decrease is of the order of 0.3–1% per day, supposing 4 h each day at room temperature and the remaining time in a refrigerator. The decrease follows the same curve for those parts of the batch stored in the refrigerator and packed later, except that the activity of the glass with the low enzyme loading decreases more rapidly for reactors that are kept at room temperature part of the day.

The sensitivity, however, has a time course completely different from that of the activity. The sensitivity remains essentially constant with time, except for a slight increase during the first day caused by losses of adsorbed enzyme. Therefore it is necessary to assume that the irreversibly inactivated urease

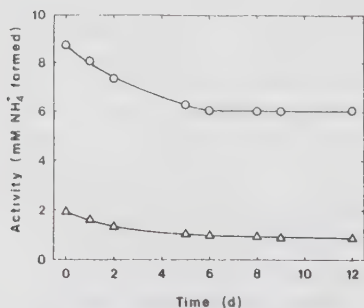


Fig. 5. Decrease in enzyme activity with time for reactors with high ($\circ$) and low ($\triangle$) enzyme loading.

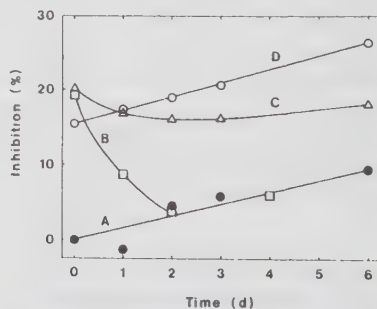


Fig. 6. Sensitivity measured with samples containing 5 nM Hg^{2+} , 10 mM Cl^- and 1 mM malic acid buffer. For explanation, see text.

TABLE 1

Measurements with different reactors prepared from the same enzyme batch. The samples were 5 ml; the solutions were 10 mM in sodium chloride and 1 mM in malic acid buffer.

Reactor	Hg ²⁺ (nM)	Inhibition (%)	Sensitivity % inhibition/pmol Hg ²⁺
1	2.5	7.5	0.600
	2.5	6.4	0.512
2	5.0	13.4	0.536
	5.0	13.1	0.524
3	7.5	19.6	0.523
	7.5	17.7	0.472
4	10.0	28.2	0.564
	10.0	27.3	0.546
5	12.5	35.1	0.562
	12.5	35.6	0.570
6	15.0	47.8	0.637
	15.0	47.0	0.627
Mean			0.556
S.d.			0.048

molecules remain bound to the glass with their mercury binding sites intact. The mercury ions will bind equally well to active and inactive molecules.

The sensitivities of reactors with low and high loading were, respectively, 0.6 and 0.3% inhibition per picomole of mercury in the presence of 10 mM sodium chloride (3.0 and 1.5% inhibition per nM Hg²⁺ for 5-ml samples). The ratios of the loadings, the activities and the inverse sensitivities were 1:3, 1:7 and 1:2.

The sensitivity of reactors packed from the same batch was almost the same for all. Table 1 shows duplicate measurements on six different reactors. The largest single source of error is the variation in the amount of enzyme packed into a reactor.

Storage

Figure 6 shows the time course of reactors with low enzyme loading. The inhibition was calculated as a percentage of the activity relative to that at the start of the experiment. A reactor without any bound mercury was used as a blank (curve A); because of the mode of calculation the activity decrease will show up as increasing inhibition. Three reactors were treated with 5 ml of 5 nM Hg²⁺ in the presence of chloride. Curve B shows the result when the reactor had been regenerated 10 min before the inhibition. Curves C and D were obtained under identical conditions except that the regeneration had been made 24 and 96 h, respectively, before the inhibition. Traces of the regeneration reagent are trapped in pores or crevices and are slowly released by diffusion resulting in a removal of mercury. If the measurements are made immediately after sampling, freshly regenerated reactor can be used;

otherwise some days should elapse between regeneration and a final rinse. If this precaution is taken (curve D) the reactors can be stored for several days before measurement, if a blank correction is applied.

The slopes of the blank and consequently of the plots for inhibited reactors are very much smaller when enzyme-glass with the higher loading is used.

Both the sensitivity and the activity of a reactor can be restored almost completely if the regeneration is done within a few hours after the inhibition. This is not the case if the reactor has been stored in inhibited condition for about a week. The inhibited fraction will be irreversibly damaged; the amount of the deactivation is 25% after 24 h and almost 100% after a week. The reactors cannot be re-used unless they are regenerated very soon after the inhibition.

Organo-mercury compounds

Organo-mercury compounds cause inhibition as shown in Table 2. The response was found to be less than that for inorganic mercury, but it is not known if the difference is real or caused by losses of the volatile compounds. The standards were checked by flame atomic absorption spectrometry. The mercury concentration in the standards were in the millimolar range but even at this level losses were observed. The problems are of course magnified at the nanomolar level used in the reactor tests.

The organo-mercury compounds behaved in a similar way as inorganic mercury and the procedure for determinations was essentially the same. Addition of chloride to the sample increased the sensitivity in the same way as for inorganic mercury but the leakage from the first to the second reactor

TABLE 2

Sensitivity of an enzyme reactor towards organo-mercury compounds

(5-ml samples were taken and the sample injection rate was about 4 ml min⁻¹. (Cl⁻) indicates that the samples were 10 mM in sodium chloride. All solutions were 1 mM in malic acid buffer.)

Sample	1st reactor inhibition (%)	2nd reactor inhibition (%)	Sum of inhibition (%)	Inhibition relative to Hg ²⁺ (%)
5 nM Hg ²⁺ (Cl ⁻)	16.4	0.8	17.2	100
10 nM Hg ²⁺ (Cl ⁻)	36.3	-0.4	35.9	100
5 nM CH ₃ HgCl	6.0	0.0	6.0	35
5 nM CH ₃ HgCl (Cl ⁻)	13.0	1.2	14.2	83
10 nM CH ₃ HgCl	16.0	1.9	17.9	50
10 nM CH ₃ HgCl (Cl ⁻)	31.1	3.8	34.9	97
5 nM Ph-HgAc	4.2	-0.4	3.8	22
5 nM Ph-HgAc (Cl ⁻)	10.3	1.2	11.5	67
10 nM Ph-HgAc	17.6	1.9	19.5	54
10 nM Ph-HgAc (Cl ⁻)	23.5	2.7	26.2	73

was somewhat larger in the presence of chloride. This indicates that complexation with chloride causes a redistribution from weak non-inhibiting binding sites to stronger inhibiting sites with the result that the sensitivity increases. It can be assumed that the ligand exchange is slower for the organo-mercury compounds than for Hg^{2+} and this is supported by the observation that the flow rate during the sampling of organo-mercury compounds had to be kept low. The somewhat larger losses to the second reactor observed indicate that the flow rate is still too high to reach equilibrium within the first reactor.

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